

## **EXHIBIT 4**

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
19 April 2001 (19.04.2001)

PCT

(10) International Publication Number  
**WO 01/27625 A1**

(51) International Patent Classification<sup>7</sup>: **G01N 33/545**,  
33/543, 33/576, 33/532, C08F 2/10, 2/50, 290/14

University, Richmond, VA 23298-0568 (US). **VALLARINO, Lidia** [US/US]; Department of Chemistry, Virginia Commonwealth University, 1001 W. Main Street, Box 2006, Richmond, VA 23284-2006 (US).

(21) International Application Number: **PCT/US00/27787**

(22) International Filing Date: 7 October 2000 (07.10.2000)

(74) Agent: **SCHWARTZ, Robert**; Law Offices of Robert M. Schwartz, P.A., Alfred I. Dupont Building, Suite 1125, 169 East Flagler Street, Miami, FL 33131-1205 (US).

(25) Filing Language: English

(26) Publication Language: English

(81) Designated States (*national*): CA, CH, DE, FI, GB, JP, US.

(30) Priority Data:  
60/158,718 8 October 1999 (08.10.1999) US

(84) Designated States (*regional*): European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

(71) Applicants and

(72) Inventors: **LEIF, Robert, C.** [US/US]; Vice President R & D, Newport Instruments, 5648 Toyon Road, San Diego, CA 92115-1022 (US). **FRANSON, Richard, C.** [US/US]; President and Treasurer, VCU-IPF, 1101 East Marshall Street, P.O. 980568, Virginia Commonwealth

Published:

— With international search report.

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: **CONJUGATED POLYMER TAG COMPLEXES**

(57) Abstract: Processes are described for: (1) the sequential solid phase synthesis of polymers with at least one tag, which can be a light emitting and/or absorbing molecular species (optical-label), a paramagnetic or radioactive label, or a tag that permits the physical separation of particles including cells. When multiple optical-labels are suitably arranged in three-dimensional space, the energy transfer from one molecular species to another can be maximized and the radiationless loss between members of the same molecular species can be minimized; (2) the coupling of these polymers to biologically active and/or biologically compatible molecules through peripheral pendant substituents having at least one reactive site; and (3) the specific cleavage of the coupled polymer from a solid phase support. The tagged-peptide or polymers produced by these processes and their conjugates with an analyte-binding species, such as a monoclonal antibody or a polynucleotide probe are described. When functionalized europium macrocyclic complexes, as taught in our U.S. patents 5,373,093 and 5,696,240, are bound to polylysine and other peptides, the emitted light increases linearly with the amount of bound macrocyclic complex. Similar linearity will also result for multiple luminescent macrocyclic complexes of other lanthanide ions, such as samarium, terbium, and dysprosium, when they are bound to a polymer or molecule.

WO 01/27625 A1

Applicants: Jingyue Ju et al.

Serial No.: 10/735,081

Filed: December 11, 2003

Exhibit 4

## CONJUGATED POLYMER TAG COMPLEXES

This invention was made with Government support under Small Business Technology Transfer Grant 5 R42 CA 73089 awarded by the National Institutes of Health, National Cancer Institute. The United States Government has certain rights in the invention.

### BACKGROUND OF THE INVENTION

#### DEFINITIONS:

To facilitate understanding of the method of this invention, the following definitions of terms used in this specification and claims are provided.

1. The term "lanthanide" is used to designate any of the trivalent lanthanide elements atomic number 57-71 as well as the lanthanide-like yttrium(III) and the actinide elements (atomic number 89-103).
2. Reactive functionality is used to mean a first atom or group capable of reacting with a second atom or group forming a covalent bond with it, as previously used in US Patents 5,373,093 and 5,696,240 to mean that both the first and second atom or group are capable of forming a covalent bond. These atom or groups include but are not limited to amines, azide, alcoholic hydroxyl, phenolic hydroxyl, aldehyde, carboxylic acid, carboxamide, halogen, isocyanate, isothiocyanate, mercapto and nitrile substituents. Functionalized alkyl, functionalized aryl-substituted alkyl, functionalized aryl, and functionalized alkyl-substituted aryl signify the respective alkyl, aryl-substituted alkyl, aryl, and alkyl-substituted aryl groups substituted with a reactive functionality.
3. Peptides are polymers that primarily are composed of monomer units that primarily are amino acids. The peptide monomer units are linked to one another by amide bonds.
4. Tag means the species or moiety that permits a molecule to be detected or to be affected non-destructively by a physical force.
5. Tagged means that a molecule that has formed a covalent bond with a tag.
6. Label means a tag that permits the detection of a molecule.

- 1 7. Labeled means that a molecule that has formed a covalent bond with a label.
- 2 8. Fluorescence means a process by which an electron of a molecule or ion that is in an
- 3 electronic singlet state (a state in which the spins of all electrons are paired) absorbs the
- 4 energy contained in a photon, with the result that this electron is elevated to a higher
- 5 energy state, and subsequently an electron of this molecule or ion loses energy in the
- 6 form of a photon and deactivates to a lower energy state. This process does not involve
- 7 a change in the spin multiplicity of the molecule or ion.
- 8
- 9 9. Luminescence means all other processes by which an electron in a molecule or ion
- 10 absorbs the energy contained in a photon, with the result that this electron is elevated to
- 11 a higher energy state, and subsequently energy is lost from an electron in the form of a
- 12 photon with the concurrent deactivation of this electron to a lower state. This process
- 13 can involve a change of the spin multiplicity of the molecule or ion.
- 14
- 15 10. Absorbance means a process by which an electron in a molecule or ion absorbs the
- 16 energy contained in a photon without the subsequent emission of a photon.
- 17
- 18 11. Optical-label means a tag capable of fluorescence, luminescence, or absorbance.
- 19
- 20 12. Luminescence-label means an optical-label that is capable of luminescence, such as a
- lanthanide macrocycle.
- 21
- 22 13. Fluorescence-label means an optical-label that is capable of fluorescence.
- 23
- 24 14. Absorbance-label means an optical-label that is capable of absorbance.
- 25
- 26 15. Other-label means a tag that is detectable by means other than fluorescence, lumines-
- 27 cence or absorption of light, or that has a specific chemical or therapeutic activity.
- 28 Other-labels include but are not limited to radioactive, paramagnetic, and sonic species.
- 29
- 30
- 31
- 32

- 1 16. Separation-tag means a tag that non-destructively affects the physical properties of  
2 molecules and molecular complexes. Separation-tags include magnetic, paramagnetic,  
3 charged, mass increasing, and density changing species.
- 4 17. Specific combining pair means a pair of molecules that form a stable complex without  
5 the formation of covalent bond(s) with one another.
- 6
- 7 18. Tagged-polymer means a polymer to which one or more tags are attached. These tags  
8 can be optical-labels, other-labels, or separation-tags.
- 9
- 10 19. Tagged-polymer-conjugate means a tagged-polymer where this polymer has formed a  
11 covalent bond with a molecular species other than itself or its tags.
- 12 20. Analyte means any compound of interest, naturally occurring or synthetic that is a  
13 member of a specific combining pair that is to be quantitated.
- 14
- 15 21. An analyte-binding species is the member of a specific combining pair that can form a  
16 stable complex with an analyte. These analyte-binding species include but are not lim-  
17 ited to:
- 18 a) an antibody or antibody fragment
- 19
- 20 (i) Such antibodies or fragments may be defined to include polyclonal antibodies  
21 from any native source, and native or recombinant monoclonal antibodies of classes  
22 IgG, IgM, IgA, IgD, and IgE, hybrid derivatives, and fragments of antibodies includ-  
23 ing Fab, Fab' and F(ab')<sub>2</sub>, humanized or human antibodies, recombinant or synthetic  
24 constructs containing the complementarity determining regions of an antibody, and the  
25 like. The methods useful for construction of all such antibodies are known to those of  
26 skill in the art.
- 27
- 28 b) a polynucleotide, polynucleotide fragment, or an oligonucleotide
- 29 (i) Such polynucleotides, polynucleotide fragments, or oligonucleotides include  
30 but are not limited to: deoxynucleic acids, DNAs; ribonucleic acids, RNAs; and pep-  
31 tide nucleic acids, PNAs.
- 32

1 c) a lectin.

2 22. Tagged-analyte-binding species means an analyte-binding species to which is attached  
3 a tag. Since competitive assays employ tagged-analytes, when tagged-analyte species  
4 are employed for a competitive assay, tagged-analyte-species should be substituted for  
5 tagged-analyte-binding species.

6  
7 23. Tagged-polymer-analyte-binding species conjugate means a polymer with one or more  
8 tags where this polymer has formed a covalent bond with an analyte-binding species.

9 1. FIELD OF THE INVENTION

10  
11 This invention concerns: Composition of matter and a process for the preparation of  
12 tagged-polymer-analyte-binding species; and the use of tagged-polymer-analyte-binding spe-  
13 cies. Tags, labels, or dyes are covalently coupled to a polymeric substrate, which is covalently  
14 coupled to an analyte-binding species. The tags include luminescent, fluorescent, and absor-  
15 bent labels or dyes; radioactive labels, paramagnetic labels; moieties that can increase the  
16 magnetic and or paramagnetic susceptibility, alter the electrical charge, alter the buoyant den-  
17 sity, and increase the mass of a polymer-analyte-binding species conjugate.

18 To facilitate the use of references in this text, the citations have been given in full at the  
19 end. The first citation in the text gives the first author's last name, year of the cited reference  
20 and the reference number preceded by Ref. in parenthesis. The (Ref. #) is always included in  
21 subsequent citations. Citations to books include the first page of the section of interest. US  
22 patents are cited by number.

23  
24 2. Description of the Prior Art

25 The sensitivity of fluorescence measurements for the analysis of biological samples is  
26 often limited by background signal due to autofluorescence or Raman scattering. For instance,  
27 a multilaboratory survey found the average autofluorescence of human lymphocytes to equal  
28 that of 657 fluorescein molecules (Schwartz et al., 1993), (Ref. 1).

29  
30 An increase in the number of conventional organic fluorescent labels per targeted site  
31 results in quenching. For example, H. M. Shapiro, 1995 (Ref. 2) p. 91 describes one attempt at  
32 amplification of fluorescence signals by Tomas Hirshfeld et al., at Block Engineering,

1 wherein several hundred fluorescein molecules were attached to a synthetic polymer, polyeth-  
2 ylenimine, which was then conjugated with antibody. The method was not successful because  
3 fluorescence emission from fluorescein molecules was quenched due to the short nearest  
4 neighbor distances between fluorophores on the same polymer molecule. See H. M. Shapiro,  
5 1995 (Ref. 2), p. 277. Presumably, this quenching is related to the partial overlap of the  
6 absorption and excitation spectra of the fluorescent molecules, J. R. Lakowicz, 1983 (Ref. 3),  
7 p. 305.

8 Haralambidis et al., (1990A) (Ref. 4) described the synthesis of both peptide-oligodeoxyri-  
9 bonucleotide and polyamide-oligonucleotide carboxyfluorescein conjugates employing an  
10 Applied Biosystems Inc. automated DNA synthesizer. The peptide or polyamide was first  
11 assembled on a solid support. The terminal amino group was converted to an amide by reac-  
12 tion with an  $\alpha,\omega$ -hydroxycarboxylic acid derivative. The free hydroxyl group was then esteri-  
13 fied with a phosphoramidite and the peptide- or polyamide-substituted polynucleotide was  
14 subsequently assembled by sequential reaction with methyl N,N-diisopropyl nucleoside phos-  
15 phoramidites. Protected lysine residues were included in both the peptide and the polyamide  
16 to provide primary amino functionalities suitable for conjugation to the fluorescent species. In  
17 a subsequent paper, Haralambidis et al. (1990B) (Ref. 5) reported labeling the polyamide-  
18 linked oligonucleotide probes with multiple carboxyfluorescein units, after deprotection of the  
19 primary amino groups of the lysine residues. However, the resulting oligonucleotides "carry-  
20 ing multiple carboxyfluorescein labels gave low levels of fluorescence due to quenching"  
21 (Ref. 5). These authors reported that "The amount of fluorescence per fluorescein moiety is 20  
22 times less than that of carboxyfluorescein in the conjugates with ten lysines", even when the  
23 lysine residues were separated by two or four spacers.  
24

25 Multiple fluorescent-labels have been bonded to dextrans in order to maximize the fluores-  
26 cence emission. Numerous fluorescent dextrans are commercially available. R. P. Haugland,  
27 1996 (Ref. 6) p. 351. Fluorescent dextrans consist of soluble dextrans (that is dextrans with a  
28 molecular mass of 3,000, 10,000, 40,000, 70,000, 500,000, and 2,000,000 daltons) conjugated  
29 with various fluorescent species such as fluorescein, dansyl, rhodamine, and Texas Red. The  
30 degrees of substitution in these fluorescent dextrans are 0.5-2 fluorescent species per dextran  
31 of 10,000 daltons, 2-4 fluorescent species per dextran of 40,000 daltons, 3-6 fluorescent spe-  
32 cies per dextran of 70,000 daltons. Conjugated dextrans are also available as so-called

1 "lysine-fixable", that is, they have incorporated lysine residues which can be used for further  
2 reaction, such as covalent attachment of antibody molecules. Fluorescein isothiocyanate  
3 (FITC) derivatives of dextran and poly-L-lysine, with degrees of substitution ranging from  
4 0.003 to 0.020 molecules of FITC per molecule of glucose and from 0.003 to 0.02 molecule of  
5 FITC per molecule of glucose, are commercially available from sources, such as Sigma-Ald-  
6 rich, 2000-2001 (Ref. 7) p. 428.

7 Siiman et al. US Patent 5,891,741(Ref. 8) have described increasing the fluorescence of  
8 individual antibody molecules by conjugation with a dextran crosslinked, ligand-(phycobilip-  
9 rotein or tandem dye) conjugates containing up to twenty five phycobiliprotein or tandem flu-  
10 orescent species per dextran molecule. US Patent 5,891,741 describes a method for preparing  
11 the antibody-aminodextran-phycobiliprotein conjugates.

12  
13 This method comprises the steps of:

14 (a) activating the antibody with iminothiolane, then purifying the activated antibody;

15  
16 (b) activating the phycobiliprotein with iminothiolane, then purifying the activated phyco-  
17 biliprotein;

18  
19 (c) combining the activated and purified antibody and phycobiliprotein;

20 (d) activating the aminodextran with sulfo-SMCC, then purifying the activated aminodext-  
21 ran;

22  
23 (e) mixing all activated components together for about 16-24 hours; and

24 (f) purifying the mixture into its components, preferably by size exclusion chromatogra-  
25 phy.

26  
27 Although US Patent 5,891,741 teaches a method to increase the fluorescence of an anti-  
28 body, it differs from the invention described below in that:

29 1) it does not describe achieving a high concentration of fluorescent labels.

30  
31 2) it does not provided a means to control the spatial organization of the labels.

32



1     3) more than one antibody molecule can be attached to an aminodextran molecule. And

2     4) the molecular weight of the aminodextran conjugate without the antibody is much larger  
3 than that of any of the following: an IgG antibody (MW, 160,000 daltons), most other com-  
4 mercial analytes, analyte-binding species, conventionally conjugated analyte-binding species,  
5 conventionally conjugated analytes, analyte-binding species conjugated with the tagged pep-  
6 tides described in this invention, and analytes conjugated with the tagged peptides described  
7 in this invention. Thus, the reaction rate of the analyte with its combining member, the amino-  
8 dextran conjugate, will be significantly slowed by being conjugated with the aminodextran.  
9

10     Peterson et al. 1998 (Ref. 9) have reported on the Merrifield synthesis of support-bound  
11 peptides that are substrates for cathepsin B and cathepsin D. These authors stated that, "The  
12 solubility properties of the PEGA support allow enzymatic permeability in an aqueous envi-  
13 ronment". The authors described PEGA as "bis(2-acrylamidoprop-y-1-yl) poly(ethylene gly-  
14 col) cross-linked dimethyl acrylamide and mono-2-acrylamidoprop-1-yl[2-aminoprop-1-yl]  
15 poly(ethylene glycol) (800)". Enzymatic cleavage liberates the peptide that is N-terminal to  
16 the cleavage site. The cathepsins were chosen because they are lysosomal endoproteases. The  
17 authors stated, "insertion of a peptide substrate between a radiolabeled chelate and its target-  
18 ing moiety (e.g., an antibody) may lead to expedited clearance of undesirable radioactivity  
19 from the liver during radioimmunotherapy and imaging". In a subsequent publication, Peter-  
20 son et al. 1999 (Ref. 10), these authors reported on the synthesis of peptides that included a  
21 site for hydrolytic cleavage by cathepsins B and D and had a DOTA group attached by a pep-  
22 tide bond to the N terminal amino acid and a p-isothiocyanatophenylalanine attached by a  
23 peptide bond to the C terminal amino acid. DOTA, which can bind the radioactive ion <sup>90</sup>Y, is  
24 an abbreviation for 1,4,7,10-tetraazacyclododecane-N, N', N'', N'''-tetraacetic acid. The p-  
25 isothiocyanatophenylalanine can be bound to the lysines of proteins including antibodies.  
26

27     These peptide conjugates: 1) are incapable of fluorescence or luminescence 2) only bind  
28 one chelating moiety; 3) are not bound to the protein via their N terminus, and 4) their mode  
29 of use does not involve enzymatic cleavage from the support.

30     Takalo et al. 1994 (Ref. 11) have reported that they were able to label IgG with up to 25  
31 europium(III) chelates per rabbit IgG and "increasing the amount of chelates in a protein does  
32 not have any major effect on quantum yield." They did note, "Accordingly, the total lumines-

1 cence can be increased by more efficient labeling as long as immunoreactivity is retained.”  
2 These authors also stated, “The most strongly reactive intermediate, dichlorotriazinyl acti-  
3 vated chelate, may also cause decreased affinities when used in high excess conditions.”  
4 Takalo et al’s disclosed chemical reactions employed for the attachment of the fluorescent or  
5 luminescent moieties are not limited to conditions that permit the retention of biological activ-  
6 ity or the retention of the chemical integrity of the biomolecule.

7 Lamture et al. 1995 (Ref. 12) have conjugated 4-(iodoacetamido)-2,6-dimethylpyridine  
8 dicarboxylate, IADP to polylysines. This polymeric conjugate of polylysine and IADP binds  
9 Tb(III) ions with very high affinity, has been coupled to proteins, and very efficiently  
10 enhances their luminescence. These authors state, “It has the added advantage that multiple  
11 luminescent Tb(III)-DPA complexes are present in each labeled protein, even if only one site  
12 on the protein is modified with the polymer, so that the molar luminescence intensity is  
13 brighter than that of conventional monomeric fluorophores.” Lamture et al. reported that  
14 attachment of the DPA to poly-L-lysine with nominal average molecular weight of 26,000  
15 results in greatly increased resistance to EDTA. They state, “These results suggest that Tb-  
16 PLDS complexes (Tb(III)-DPA poly-L-lysine conjugates) are approximately 50,000 times  
17 more stable than Tb-EDTA.”  
18

19 The conjugation of bovine serum albumin, BSA, to Tb-PLDS complexes was described.  
20 The unreacted lysines of the DPA poly-L-lysine conjugates were reacted with N-hydroxysul-  
21 fosuccinimide in the presence of 1-(3-dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride,  
22 EDC. After the lysines were activated, BSA was added. Similar conditions were  
23 employed to conjugate ovalbumin, protein A, and avidin. Coomassie-Blue stained Sodium  
24 DodecylSulfate-polyacrylamide electrophoresis, SDS-PAGE, of the BSA conjugates showed  
25 the presence of a continuum of molecular weights starting with BSA monomers. The distribu-  
26 tion of terbium luminescence on the gels was not mentioned or reported. In the case of the avi-  
27 din conjugates, Lamture et al. stated that it would be possible to obtain better results “by  
28 protecting lysines essential for biotin binding during the labeling reaction.” These authors  
29 employed only one type of reactive functionality, the epsilon amino group of lysine, rather  
30 than the two or more reactive functionalities, as specified in the present invention. All of the  
31 chemistries occurred in the liquid phase, rather than with the use of a support as specified in  
32 the present invention.

1 Kwiatkowski et al. 1994 (Ref. 13) have compared the emissions from 20 base long oligo-  
2 nucleotides that additionally included "either 1, 2, 5, 10, or 20 europium chelate-modified  
3 nucleotides". These authors stated, "that the direct fluorescence, per europium ion, is indepen-  
4 dent of the number of chelates present in each oligonucleotide." They concluded that the  
5 emission intensity could be increased in the proportion of the number of chelates added. The  
6 oligonucleotides were labeled by chemically adding deoxyuridine and deoxycytidine deriva-  
7 tives onto either the 5'- or the 3'-end of oligonucleotides. Addition to the 3'-end permitted the  
8 use of standard DNA supports. The deprotection steps included 0.1M sodium hydroxide and  
9 "standard ammonia deprotection". These steps are inconsistent with the maintenance of bio-  
10 logical function of proteins, such as antibodies. The lanthanide binding functionality is  
11 directly attached to the analyte-binding species.

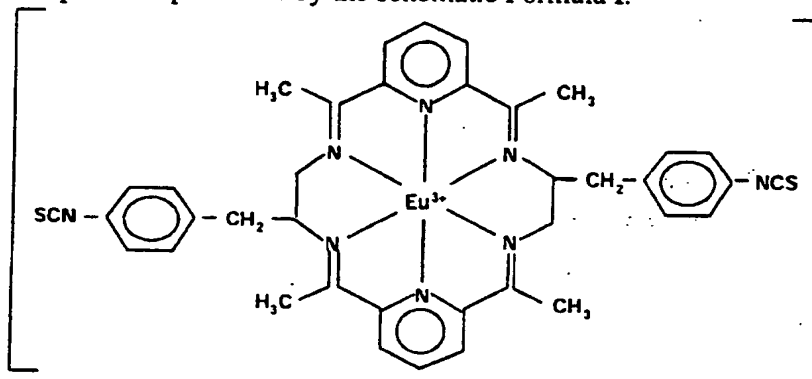
12 Salo et al. 1998 (Ref. 14) have synthesized disulfide linkers for the solid phase synthesis of  
13 oligonucleotides. The disulfide linker N-[16-[(4,4'-Dimethoxytrityl)oxy]-  
14 12,13dithiahexadecanoyl] was attached to amino-modified Tentagel. "The protected oligonu-  
15 cleotides were assembled on an Applied Biosystems 392 DNA synthesizer" using phospho-  
16 midites. The first two nucleotides were N<sup>4</sup>-(6-aminohexyl)-2'-deoxycytidine, which were  
17 both labeled with either 5-(dimethylamino)-1-naphthalenesulfonyl chloride or a dichlorotriaz-  
18 ine derivative of a photoluminescent europium(III) chelate. The europium(III) labeled chelate  
19 18mer oligodeoxyribonucleotide was cleaved from the solid by dithiothreitol and was used  
20 successfully for a sandwich hybridization.  
21

22 The methodology of the present invention differs from Salo et al. (Ref. 14) because 1) the  
23 species produced by these authors could not be stored for subsequent attachment of an oligo-  
24 nucleotide without the use of specialized, expensive instrumentation. 2) Their methodology  
25 was unsuited and not directed to proteins or other analyte-binding species. 3) Enzymes were  
26 not used for the cleavage of their oligonucleotides from the support, and 4) No mention was  
27 made of the possibility of employing the disclosed technology with peptides or PNAs.

28  
29 Inorganic phosphor particles (D. A. Zarling et al. US Patent 5,736,410, 1998 (Ref. 15) have  
30 been used as multiple labels or tags. However, the absorption spectrum of these particles is  
31 narrow, resulting in the preferred method of illumination being two photon absorption of  
32 infrared laser light. The use of these particles is limited by nonspecific binding; furthermore,

1 the total binding of rigid particles to solid substrates and cells is limited to a small contact  
2 zone

3 Vallarino and Leif have reported in US Patent 5,373,093, 1994 (Ref. 16) and its Continua-  
4 tion-In-Part US Patent 5,696,240, 1997 (Ref. 17) on symmetrically di-functionalized water  
5 soluble macrocyclic complexes of rare-earth, actinide and yttrium ions. A di-functionalized  
6 macrocyclic complex is represented by the schematic Formula I:



15 Formula I

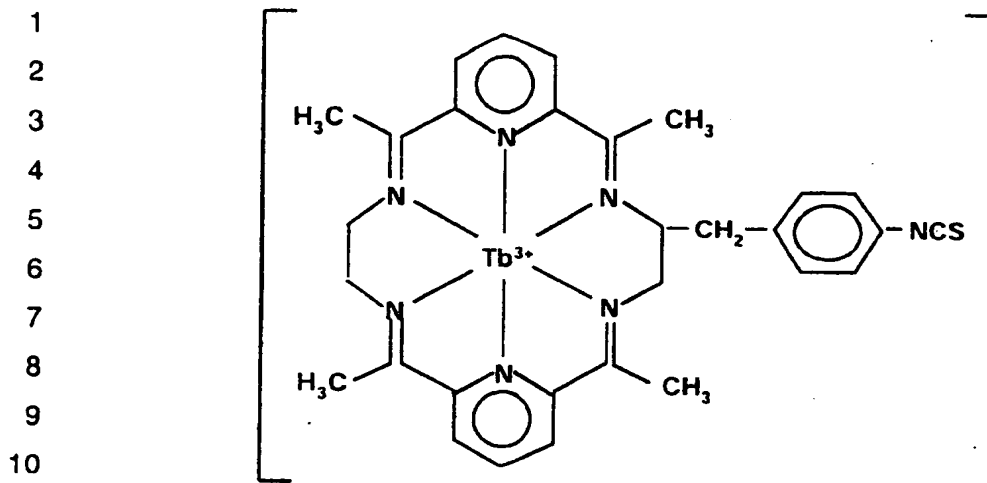
16  
17 Formula I is the di-isothiocyanate derivative having the structure shown in column 10 of  
18 US Patent 5,373,093. Specifically, it is one of the isomers of the cationic europium macrocy-  
19 clic moiety containing a 4-isothiocyanate-benzyl- substituent on each of the aliphatic side-  
20 chains. The molecular formula of the moiety is  $C_{38}H_{36}N_8S_2Eu$ . Its trichloride was used in liq-  
21 uid phase coupling reactions of this application.

22  
23 In US Patent 5,696,240, asymmetrically mono-functionalized water soluble macrocyclic  
24 complexes of rare-earth, actinide and yttrium ions are described. A mono-functionalized mac-  
25 rocyclic complex is represented by the schematic Formula II:

26 Formula II is the mono-isothiocyanate derivative having the structure shown in Claim 13  
27 of US Patent 5,696,240. Specifically, it is the cationic terbium macrocyclic moiety containing  
28 a 4-isothiocyanate-benzyl-substituent on one of the aliphatic side-chains. The molecular for-  
29 mula of the moiety is  $C_{30}H_{31}N_7STb$ . Its trichloride was used in solid phase coupling reactions  
30 of of this application.

31

32



Formula II

The following abbreviations will be used to describe molecular structures related to those shown in Formula I and in Formula II. Any and all of the metal ions selected from the group consisting of a lanthanide having atomic number 57-71, an actinide having atomic number 89-103 and yttrium(III) having atomic number 39 will have M as their abbreviation. Specific metal ions will be given as their standard chemical symbols. The mono-functionalized and di-functionalized macrocyclic complexes will be abbreviated respectively as "Mac-mono" and "Mac-di". The term Mac without the mono or di prefix will include both the mono-functionalized and di-functionalized macrocyclic complexes (Mac-mono and Mac-di). When a specific peripheral pendant substituent having at least one reactive site (reactive functionality) is specified, its abbreviation will be given as a suffix. Thus, the compound shown in Formula I is abbreviated as EuMac-di-NCS. The compound shown in Formula II is abbreviated as TbMac-mono-NCS. The generic term, M-Mac, will refer to any and all of the macrocyclic species covered by US patents 5,373,093 and 5,696,240.

US Patent 5,373,093 and its Continuation-In-Part US Patent 5,696,240 teach the structures, synthesis and use of functionalized water soluble macrocyclic complexes of lanthanide, actinide and yttrium ions. "Symmetrically di-functionalized water soluble macrocyclic complexes of lanthanide, actinide and yttrium ions were obtained by metal templated, Schiff-base, cyclic condensation of: (1) a functionalized 1,2-diaminoethane and a dicarbonyl compound selected from the group consisting of 2,6-dicarbonylpyridine, 2,6-diformylpyridine, 2,5-dicarbonylfuran, 2,5-diformylfuran, 2,5-dicarbonyl-thiophene and 2,5-diformylthiophene; or (2)

1 1,2-diaminoethane and a ring-substituted heterocyclic dicarbonyl compound selected from a  
2 group consisting of substituted 2,6-dicarbonylpyridine, substituted 2,6-diformylpyridine, sub-  
3 stituted 2,5-dicarbonylfuran, substituted 2,5-diformylfuran; substituted 2,5-dicarbonyl  
4 thiophene, and substituted 2,5-diformylthiophene."

5 US Patent 5,696,240 teaches the structures, synthesis and use of "asymmetrically function-  
6 alized water soluble macrocyclic complexes of the lanthanide, actinide and yttrium ions were  
7 obtained by metal templated, Schiff-base, cyclic condensation of appropriately substituted  
8 diamine and dicarbonyl precursors, with such precursors contributing two heteroaromatic  
9 moieties (pyridine, furan, thiophene, or a combination thereof) to the resulting macrocyclic  
10 structure. The coordination complexes thus formed are kinetically stable in dilute aqueous  
11 solution. They are further reacted, or coupled, through a substituent on the 1,2-diaminoethane  
12 or on the pyridine, furan, or thiophene moieties, to one of the following: proteinacious materi-  
13 als, polysaccharides, polynucleotides, peptide nucleic acids, other biologically compatible  
14 macromolecules or bridging molecules which, can be further reacted or coupled to the above  
15 mentioned substrates. These macrocyclic complexes are suitable in the preparation of reporter  
16 molecules and for magnetic resonance, radiation imaging and radiation therapy."  
17

18 Leif et al. 1994 (Ref. 18) described the use of symmetrically di-isothiocyanate-functional-  
19 ized macrocyclic complexes of a lanthanide(III) ion, which served as the light-emitting center.  
20 The isothiocyanate functionalities allow covalent coupling of the lanthanide(III) macrocycles  
21 to a biosubstrate. The Eu(III) and Tb(III) complexes possess a set of properties -- water solu-  
22 bility, inertness to metal release over a wide pH range, ligand-sensitized narrow-band lumi-  
23 nescence, large Stoke's shift, and long excited-state lifetime -- that provide ease of staining as  
24 well as maximum signal with minimum interference from background autofluorescence.  
25 These authors stated, "The results with the  $^5D_0 \rightarrow ^7F_2$  (610-625 nm) Eu(III) transition,  
26 which is the major signal source, show that the luminescence of the EuMac-enhancer system  
27 is highly dependent upon the choice of both buffer and solvent. The emission intensity  
28 increases dramatically in the absence of those buffers that contain anions, such as carbonate,  
29 capable of competing with the  $\beta$ -diketonate enhancers as ligands for Eu(III). The emission  
30 intensity also increases greatly in the less hydroxylic solvents. However, vibrational deactiva-  
31 tion by interaction with the -OH groups of solvent molecules can not be solely responsible for  
32

1 the energy loss, since substitution of D<sub>2</sub>O for H<sub>2</sub>O as the solvent had been reported (Ref. 19)  
2 to result only in a three-fold increase of the EuMac excited-state lifetime.”

3  
4 The low quantum yield of the EuMac in aqueous medium probably precludes its use as an  
5 optical-label for the observation and measurements of live cells (Ref. 18). However, this com-  
6 plex can be used in conventional fluorescence (luminescence) microscopy, providing the cells  
7 are mounted in the appropriate nonaqueous medium or in an aqueous medium to which has  
8 been added a micellar solution which contains a second lanthanide ion, Bromm et al. 1999  
9 (Ref. 20) and Quagliano et al. 2000 (Ref. 21). In the case of a nonaqueous medium (Adeyiga  
10 et al. 1996 (Ref. 22), either ethyleneglycol replaces glycerol, which is conventionally  
11 employed as the mounting medium, or a permanent mounting medium, such as ACCU-  
12 MOUNT 60 (Stephens Scientific, Riverdale, NJ), is employed. A dry specimen can be either  
13 observed and/or quantitated. Clinical diagnostic and other uses of the EuMac as optical-label,  
14 such as immunodiagnosics, are feasible providing the measurements are performed in a non-  
15 aqueous solvent such as ethanol or the sample is dry.

16 Adeyiga et al. 1996 (Ref. 22) described: 1) Protocols for the coupling of NCS-substituted  
17 Eu-macrocycles to proteins and for the mounting on microscope slides of particles labeled  
18 with luminescent Eu-macrocycles. The emission/excitation spectra of the dried, slide-  
19 mounted particles were investigated. 2) The synthesis of lanthanide-macrocycles having a sin-  
20 gle peripheral functionality, as well as the structure and properties of the complexes.

21  
22 The mono-isothiocyanate functionalized macrocyclic complex of Tb(III) (Ref. 17), which  
23 is illustrated in Formula II of this application, and the di-substituted analog (Ref. 16) illus-  
24 trated in Formula I of this application, fulfill all fundamental requirements of a luminescent  
25 marker for cell imaging and solid-phase immunoassays. These complexes do not release the  
26 lanthanide ion even in very dilute aqueous solution and the presence of competing ligands.  
27 Since the lanthanide macrocyclic complexes are formed around the lanthanide ions during the  
28 lanthanide-templated synthesis, rather than by binding the lanthanide ions to preformed mac-  
29 rocyclic ligands, these species are kinetically stabilized (Ref. 23) and will not dissociate under  
30 the experimental conditions employed for the formation of antigen-antibody complexes or for  
31 the hybridization of an oligonucleotide to DNA or RNA. As is well known, the lanthanide(III)  
32 ions in the M-Mac can bind two enhancers, one on each of the opposite sides of the macrocy-

1 cle (Ref. 23). This binding permits an efficient energy transfer from the absorber—the  
2 enhancer—to the lanthanide emitter. The enhancers also shield the excited lanthanide ion  
3 from direct contact with water, which ordinarily would quench the luminescence by vibronic  
4 interaction.

## 5 6 SUMMARY OF THE INVENTION

7  
8 In accordance with this invention, there is provided

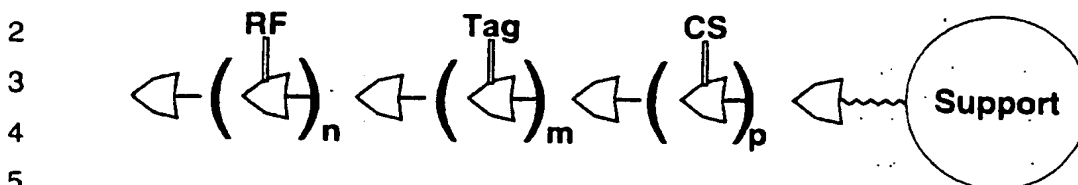
9 a tagged, water-soluble, polymer linked to a solid support and selectively cleavable there-  
10 from, such polymer comprising a cleavage segment of known composition, form, and  
11 sequence within which cleavage of a bond separates the polymer from the support, a second  
12 segment of known composition, and sequence comprising one or more tagged monomer units;  
13 a third segment comprising one or more monomer units with a reactive functionality that can  
14 form a covalent bond with an analyte-binding species; and zero or more spacer monomer  
15 units, wherein at least one of the tagged monomer units is linked to a moiety that is an optical-  
16 label, an other-label or a separation-tag. These tags can serve as a luminescent, fluorescent,  
17 and/or absorbent label; or as an other-label, which serves as a radioactive, paramagnetic, or  
18 sonic label; or as a separation-tag that non-destructively affects a physical property, such as  
19 magnetic susceptibility, electrophoretic mobility, buoyant density or mass, of a specific com-  
20 bining pair or species of which the analyte is a part. Emission of light can take place by a  
21 luminescence or fluorescence mechanism as defined. The absorption and/or emission of light  
22 by the optical-label can occur in the range from 200 to 1,400 nanometers. Other-labels can  
23 also be radioactive, capable of being transformed into radioactive substances, and/or detect-  
24 able by radiological means including but not limited to radioactive emissions and/or magnetic  
25 resonance imaging. The binding of multiple separation-tags, non-destructively affecting a  
26 physical property can sufficiently change such property to permit the separation of a specific  
27 combining pair or species of which the analyte is a part. Any tag can serve multiple purposes.  
28 For example, lanthanides can be luminescent, paramagnetic, as well as radioactive; lan-  
29 thanides can change the charge, buoyant density and mass of tagged-polymer-analyte-binding  
30 species.

31

32



1 The polymer according to the invention can be represented by Formula III:



7 in which each left pointing broad-arrow shape represents a monomer unit; RF represents a  
8 reactive functionality linked to a monomer unit; Tag independently at each occurrence repre-  
9 sents an optical-label, or other-label, or separation-tag linked to a monomer unit; CS repre-  
10 sents at least one monomer unit constituting the cleavable link to the support shown by the  
11 circular shape at the right; broad-arrow shapes without other indication represent spacer  
12 monomer units, n is a number from 1 to 10, m is a number from 1 to 1,000 and p is a number  
13 from 1 to 25.

14 The first monomer unit of the polymer is covalently bound to the support or to another  
15 polymer attached to the support. The number of spacer monomers is governed by cost and  
16 depends on their position in the polymer; it can reasonably range from 0 to  $(20 \times m) + 100$ .  
17 Spacer monomers can be placed within groups of both tag-bearing and reactive functionality-  
18 bearing monomers. From 1 to 10 types of tags can be linked to monomer units.

19  
20 The molecular weight of the polymer of this invention is at least that of the essential three  
21 monomer units defined above. There is in principle no upper limit except the practical consid-  
22 eration that the added cost of more monomer units be justified by added benefits of their pres-  
23 ence. Hence the polymer of the invention preferably includes from 3 to 1000 monomer units  
24 and more preferably has a molecular weight in the range from 1000 to 100,000 daltons. The  
25 polymer of the invention, therefore, can have bound one to approximately 1,000 tags; it can be  
26 selectively cleaved from the support by enzymatic as well as other techniques that do not  
27 destroy the tags; it can be covalently bound to an analyte-binding species or an analyte; and it  
28 can be so cleaved after being bound to this analyte-binding species or analyte.

29  
30 The linkage of the polymer to a solid support permits monomer units to be added in a spe-  
31 cific order, suitably by an iterative synthesis. Thus, in the case of peptides or any other type of  
32 polymer in which specific monomer sequences permit tags to have a specified relative geo-

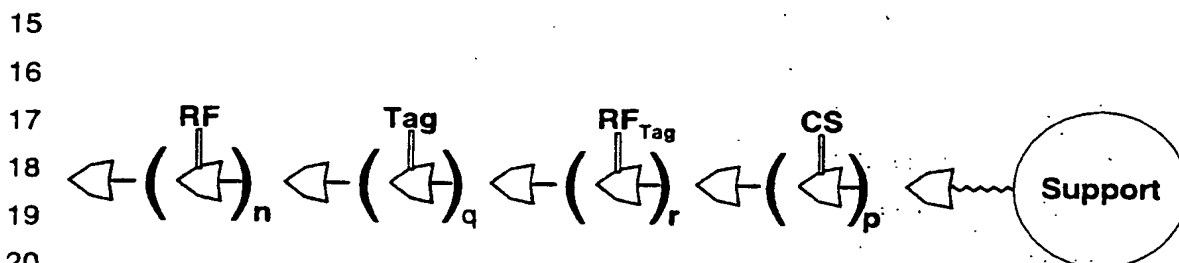
1 metric position in space, these geometric relative positions can be controlled. This eliminates  
2 the often very difficult synthetic chemistry problem of synthesizing a direct bond between two  
3 molecules and also providing a reactive functionality that can couple this dimer to an analyte-  
4 binding species. Thus, a pair of molecules where one transfers energy to the other can be  
5 linked together by each separately forming covalent bonds with monomers that are part of the  
6 same polymer or monomers with appropriate optical-labels being directly incorporated into  
7 the polymer. A further advantage is the selective cleavage of the polymer from a solid support,  
8 which provides the ability to work with the polymer attached to solid support or in solution, as  
9 desired. Selective cleavage means the ability to sever the linkage between the polymer and the  
10 solid support, in preference to severing covalent linkages of monomer units within the poly-  
11 mer or linkages of tag moieties to monomer units, or linkages between the polymer and ana-  
12 lyte-binding species or analyte. The formation of a complex between the analyte-binding  
13 species and an analyte where one, or the other, or both are an optical-labeled-polymer-conju-  
14 gate permits the detection and/or quantitation of this analyte by the interaction of light with  
15 the light absorbing and light emitting species of the water-soluble polymer; or the detection or  
16 use of other-labels; or the separation of this analyte or specific combining pair by physical  
17 means. After cleavage of the tagged-polymer-conjugate from a support, either the detection  
18 and/or quantitation of an analyte and/or the separation of an analyte or specific combining pair  
19 by physical means can be performed in solution. Yet another possible use is the directed deliv-  
20 ery of the tags to cells for therapeutic purposes.

21 This invention addresses the deficiencies in the prior art by providing a series of peptides  
22 or other polymers that contain covalently bound tags, a reactive functionality for coupling to  
23 an analyte-binding species, and a cleavable linkage to a solid support. Procedures for produc-  
24 ing tagged-polymer-analyte-binding species are also described. The possible tags include  
25 optical-labels, other-labels, and separation-tags while both these tags and the analyte-binding  
26 species may be labile, any potential danger of decomposition under the conditions required for  
27 the chemical reactions involved in the sequential solid-phase synthesis of polymers and in the  
28 cleavage of these polymers from the solid support is minimized according to the invention.  
29 The preparation of tagged-analyte-binding species is simplified for the end user according to  
30 the invention, when the analyte is bound in the solid phase to a pre-manufactured tag and then  
31  
32

1 the tagged-analyte-binding species is selectively cleaved from the support with its intact tag(s)  
2 attached.

3 This invention also includes a water-soluble polymer linked to a solid support and selec-  
4 tively cleavable therefrom, comprising closest to the support a cleavage segment, of known  
5 composition and sequence made up of at least one monomer unit; a second segment of known  
6 composition and sequence separated from the support by at least the cleavage section and  
7 including one or more monomer units each of which is linked to either a reactive functionality  
8 able to be covalently coupled to a tag or linked to a tag, and a third segment of known compo-  
9 sition and sequence separated from the support by at least the cleavage section and including  
10 at least one monomer unit linked to a reactive functionality, capable of forming a covalent  
11 bond with an analyte-binding species or an analyte; from 1 to 10 types of tags can be linked to  
12 the monomer units.

13  
14 Such a polymer can be represented by the schematic Formula IV:



22 wherein each left pointing broad-arrow shape represents a monomer unit; RF independently  
23 represents a reactive functionality linked to a monomer unit and serving to bind to an analyte-  
24 binding species; RF<sub>tag</sub> independently at each occurrence represents a reactive functionality  
25 able to be covalently coupled to a tag; Tag independently at each occurrence represents an  
26 optical-label, or other-label, or separation-tag linked to a monomer unit; CS represents at least  
27 one monomer unit constituting the cleavable link to the support shown by the circular shape at  
28 the right; broad-arrow shapes without other indication represent spacer monomer units, which  
need not be present; n is a number from 1 to 10; r is a number from 0 to 1,000, q is a number  
from 0 to 1,000, provided that the sum of r and q is a number from 1 to 1,000; and p is a num-  
ber from 1 to 25.

## 29 30 DETAILED DESCRIPTION OF THE INVENTION INCLUDING PREFERRED 31 EMBODIMENTS 32

1 Three ways to covalently bind a tag with special desired properties to a polymer back-bone  
2 are: 1) Synthesize monomers which incorporate the tag(s); if necessary, each tag can be chem-  
3 ically protected by an appropriate protecting group. The tagged monomers are then incorpo-  
4 rated into the polymer in the desired order as the polymer is synthesized. An example of a  
5 commercially available (AnaSpec, Product # 23357, 2000-2001) tagged monomer is Fmoc-  
6 Lys(Fluorescein)-OH. 2) Sequentially react a growing polymer, after the addition of a func-  
7 tionalized monomer, with a species capable of forming a bond with the reactive functionality  
8 of said monomer, with the result of producing a tagged monomer already incorporated into the  
9 polymer. 3) Synthesize a polymer containing various monomer units with different reactive  
10 functionalities, and react these with species specific for each functionality to produce tagged  
11 monomer units. These reactions can occur after all of the monomers have been incorporated  
12 into the polymer, with the advantage that the tags are never exposed to the conditions required  
13 for the reactions employed in the polymer synthesis. If the polymer is synthesized on a solid  
14 support, there is still the possibility that the tags may be affected by the often harsh conditions  
15 required for the cleavage of the polymer from the support. This potential problem is further  
16 exacerbated if the binding of the analyte-binding species to the tagged-polymer is carried out,  
17 as often desirable, while the polymer is still bound to the solid support. This invention there-  
18 fore includes a very mild enzyme-based selective cleavage of the polymer from the solid sup-  
19 port, carried out under conditions that do not affect either the tags or the analyte-binding  
20 species. In fact, a protein (an antibody) has been demonstrated to withstand the cleavage step.  
21 This invention has the further advantages of permitting control of the location of the tags rela-  
22 tive to each other, and of requiring reaction with only one site on the analyte-binding species,  
23 thus minimizing interference with its biological function.

24 A more detailed description of the elements of the tagged-polymer-analyte-binding species  
25 and their individual and combined utility follows.  
26

## 27 **Water-Soluble Tagged-Polymer Linked to a Solid Support**

### 28 **Solid Support**

29 The solid support is any water-insoluble solid, organic or inorganic, that can be linked to a  
30 polymer comprising at least one tagged monomer unit, at least one monomer unit bearing a  
31 reactive functionality, and at least one monomer unit that can be selectively cleaved from the  
32 support, and when desired at least one spacer monomer unit.

1 The criteria for the use of such a support, first disclosed in R. B. Merrifield's pioneer publi-  
2 cation (Ref. 24) on solid phase peptide synthesis, are still applicable here. This technology is  
3 extensively described in P. Lloyd-Williams et al. 1997 (Ref. 25), which is incorporated by ref-  
4 erence.

5 Preferably the support is a swellable bead with pendant hydrophilic polymer side chains  
6 having a wet particle size of about 10 to 1,000 microns, functionalized so as to react with the  
7 terminal monomer of the cleavage segment. In the case of a peptide cleavage segment linked  
8 to the support through a terminal carboxyl group, the bead is functionalized with a group reac-  
9 tive therewith, such as an amino group or a halomethyl group, and in the case of a polynucle-  
10 otide, an aliphatic hydroxyl. The optimum size of the beads will depend on the exact  
11 circumstances of their utilization including cost. Presently, it is beads in the range of 150-300  
12 um.

13  
14 Suitable solid supports are known in the art and many are commercially available. Exam-  
15 ples are listed in the Polymer Laboratories Catalog 2000 and the associated internet URL  
16 ([www.polymerlabs.com](http://www.polymerlabs.com)) which are here incorporated by reference. The supports can be  
17 hydrophobic or hydrophilic. When the support is hydrophobic, the polymer is bonded to the  
18 support in the presence of an organic solvent that swells the support to a multiple of its dry  
19 volume. Hydrophobic supports include: cross-linked polystyrene, chloromethyl-substituted  
20 polystyrene, aminomethyl-substituted polystyrene with controlled degree of crosslinking as  
21 with approximately 1% divinylbenzene, and polyamide. A hydrophilic support has the advan-  
22 tage that an organic solvent is not required and the polymer can be bonded to the support in  
23 the presence of water. Hydrophilic polymers such as polyethylene glycol can be grafted to  
24 hydrophobic supports such as polystyrene. In the resulting structure, the hydrophobic compo-  
25 nent of the support provides mechanical stability while the hydrophilic component increases  
26 the number of sites that can be employed for polymer synthesis, which is proportional to the  
27 number of polymers that can be synthesized. Hydrophilic monomer units of any desired  
28 molecular size can serve to increase the length of the cleavable link of the polymer of the  
29 invention to the solid support. This increased access to these polymers facilitates the addition  
30 of monomer units, the reactions with other molecules, and in particular the interaction of an  
31 enzyme with its substrate in selectively cleaving the polymer of the invention from the sup-  
32 port. Other suitable hydrophilic supports include polyvinyl alcohol bound to acrylic polymers

1 and, in general, any hydrophilic polymer that does not interfere with the chemical reactions of  
2 the Merrifield synthesis, and that permits an amino acid or other monomer to be bound to the  
3 support via a cleavable covalent link and to be cleaved therefrom. These supports are  
4 described in Lloyd-Williams et al. 1997 (Ref. 25) Chapter 2, Solid-Phase Peptide Synthesis,  
5 2.1 The Solid Support p 19.

6 Preferably, the support is a hydrophilic bead with pendant hydrophilic polymer side chains  
7 that has an exceptionally high swell in all solvents including water, and can allow large mac-  
8 romolecules, such as enzymes, to permeate the particles. The end of the polymer side chains  
9 distal to the bead should be a reactive functionality, reacting with a reactive functionality of  
10 the monomer reactant, in the way that an amino group linked to the support reacts with a car-  
11 boxyl functionality of an amino acid in forming a peptide.

#### 13 **Water Soluble Tagged-Polymers**

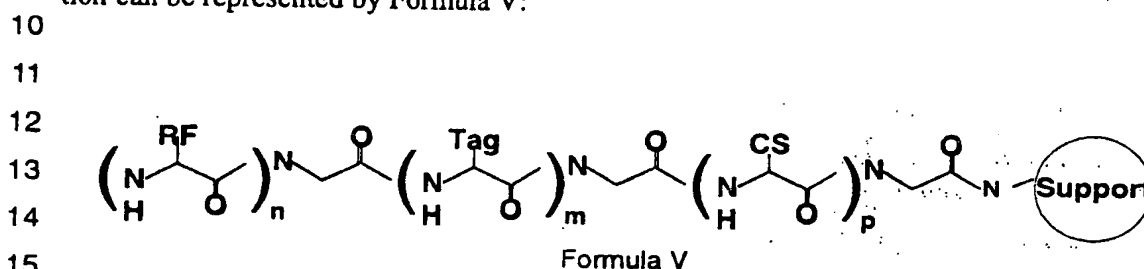
14 Polymers provided according to this invention include all structures available through iter-  
15 ative synthesis including polypeptides, nucleic acids, oligosaccharides; and in general any lin-  
16 ear polymer containing tagged monomer units and terminating at one end with functional  
17 group(s) suitable for binding to a solid support; and while at the other end a functional group  
18 is available that is suitable for binding to it another monomer including a monomer with a  
19 reactive functionality that can form a covalent bond with a member of an analyte-combining  
20 pair. A variant on this is to form dendrimeric structures which include the polymer of the  
21 invention within a branched polymeric structure.

22 Tagged monomer units in the polymer according to this invention are monomer units that  
23 include an optical-label, other-label, or a separating-tag. Monomer units with a reactive func-  
24 tionality covalently bind with a member of a specific combining pair, usually the analyte-  
25 binding species, while not reacting with the species that constitute or form tags on the tagged  
26 monomers. Spacer monomer units are those that lack either a tag or a reactive functionality.  
27 Many types of monomer units are available; preference is given to those that can participate in  
28 iterative syntheses of polymers according to the invention in which the kind, number, and  
29 order of the monomer units follows a predetermined pattern, and for which the spatial geomet-  
30 ric orientation of the resulting polymers can be ascertained.

31  
32

1 As pioneered by Merrifield (Ref. 24), such iterative syntheses are preferably carried out  
 2 with the first monomer unit linked to a solid support, either directly, or through one or more  
 3 monomer units not intended to be part of the polymer according to the invention; the subse-  
 4 quent monomer units are then successively linked to the first monomer unit in stepwise fash-  
 5 ion, until the predetermined kind and number of monomer units have been linked in the  
 6 desired order, whereupon the polymer so formed is selectively cleaved from the support.

7 When the polymer according to the invention includes a polypeptide, the monomer units  
 8 comprise aminocarboxylic acid units, amino acids. The polypeptide according to the inven-  
 9 tion can be represented by Formula V:



17 In Formula V, the free amino group end of the peptide is at the left and the carboxylic acid  
 18 end is at the right; the solid support is shown by the circular shape at the far right. RF repre-  
 19 sents a reactive functionality of an amino acid; Tag independently at each occurrence repre-  
 20 sents an optical-label, other-label, or separation-tag covalently bound to an amino acid; CS  
 21 represents a cleavable link to the support. Spacer amino acids, which have unreactive side  
 22 chains, are shown as lacking a side chain. n is a number from 1 to 10, m is a number from 1 to  
 23 1,000, and p is a number from 1 to 25.

24 From 1 to 10 types of tags can be linked to the amino acid monomer units. At least one  
 25 amino acid with a reactive functionality is required to bond to one analyte-binding species.  
 26

27 The molecular weight of the polymer of this invention is at least that of the essential three  
 28 monomer units defined above. There is in principle no upper limit except the practical consid-  
 29 eration that the added cost of more monomer units be justified by added benefits of their pres-  
 30 ence. Hence the polymer of the invention preferably includes from 3 to 1000 monomer units  
 31 and more preferably has a molecular weight in the range from 1000 to 100,000 daltons.  
 32

1 The first amino acid in the polymer sequence according to the invention is covalently  
2 linked to the support directly or through a group not part of the polymer of the invention  
3 attached to the support. The number of spacer amino acids is governed by a balance between  
4 the cost of synthesis, which increases with the number of spacers, and the optimal number of  
5 spacers required to provide the desired three-dimensional conformation of the peptide. The  
6 number of spacer amino acids can reasonably range from 0 to  $(20 \times m) + 100$ . Spacer amino  
7 acids can be intercalated as appropriate within or between groups of both tag-bearing and  
8 reactive functionality-bearing amino acids.

9 The amino acids functioning as monomer units in the polymer according to the invention  
10 can be either naturally occurring or synthetic; they can be alpha amino acids or other com-  
11 pounds that contain at least one amino group and at least one carboxyl group. The amino acids  
12 suitable for coupling to a tag or forming a covalent linkage to an analyte-binding species are  
13 N-terminal amino acids with free amino groups and those amino acids that have side chains  
14 carrying reactive functionalities such as: amino groups, carboxyl groups, hydroxyl groups,  
15 and mercapto groups. The chemistry of these and other coupling reactions is described in Her-  
16 manson, 1996 (Ref. 26) which is incorporated by reference. All naturally occurring alpha  
17 amino acids except glycine are in the L configuration and have synthetic D counterparts.  
18 Many synthetic amino acids, both non-functionalized or functionalized, have been synthe-  
19 sized as racemates as well as the L and D forms and can be incorporated into peptides by the  
20 method of iterative synthesis. Some of these are described in Barrett and D. T. Elmore, 1998  
21 (Ref. 27) which is here incorporated by reference. Numerous 9-Fluorenylmethyloxycarbonyl  
22 (Fmoc) and t-Butoxycarbonyl (Boc) amino acids including those with protected reactive func-  
23 tionalities are commercially available. Examples are listed in the AnaSpec Catalog 2000-  
24 2001, which is here incorporated by reference. A reactive functionality can be introduced into  
25 a non-functionalized and/or functionalized amino acid by the methods and reagents described  
26 by Hermanson 1996 (Ref. 26) Part II, Bioconjugate Reagents p. 169.  
27

28 Examples of amino acids carrying a reactive functionality are the N-terminal amino acid  
29 with a free amino group, which can react with iodoacetic acid, and to which a protein can be  
30 linked, and amino acids that include two or more amino groups, two or more carboxyl groups,  
31 sulfhydryl groups, hydroxyl groups, halogen groups, aldehyde groups, alkenes, alkynes, thio-  
32 cyanates, isothiocyanates, and ethoxide groups. The polypeptide according to this invention



1 can include two or more different monomer unit amino acids with reactive functionalities,  
2 such as lysine and cysteine, and can include two or more different spacer monomer unit amino  
3 acids, such as alanine, glycine, proline, tryptophan, and homocysteic acid.

4 While participating in the iterative synthesis of the polymer of the invention, the functional  
5 groups in the functionalized monomer units can be protected with a suitable protective group  
6 that is subsequently removed. Suitable protective groups include benzyl, benzyloxycarbonyl,  
7 and ring substitution products thereof; t-butyl and t-butoxycarbonyl; 9-fluorenylmethoxycar-  
8 bonyl, o-nitrophenylsulfenyl, 3-nitro-2-pyridinesulfenyl and dithiasuccinoyl. While so pro-  
9 tected, the functional groups in the functionalized monomer units are preserved from reacting  
10 with reagents affecting other groups in the molecule; when it is desired that these functional  
11 group react, the groups are deprotected by reaction with an appropriate agent under the mild-  
12 est possible conditions. Suitable deprotecting methods conditions include heating, catalytic  
13 hydrogenation, hydrolysis assisted by acid or base, and thiolysis or reductive exchange of a  
14 disulfide protecting group with a reagent containing a sulfhydryl group.  
15

16 It is well known that the inclusion of even a single unit of certain amino acids can termi-  
17 nate an alpha helix or beta pleated sheet. This occurs because the introduction of one of these  
18 amino acids can result in a drastically different molecular geometry and consequent relative  
19 orientations of neighboring monomer units. Amino acids capable of changing the secondary  
20 structures of peptides include but are not limited to: one or more D-alpha-aminocarboxylic  
21 acid or proline monomer units. Where this effect is desired, for example in order to provide a  
22 more favorable orientation of the two members of a light emitting/light absorbing energy  
23 transfer pair, D-alanine and/or proline can be included in the polymer as spacer monomer  
24 units. A simple change in the number of amino acids between two tagged amino acids can sig-  
25 nificantly change their relative position (L. Pauling, 1960 (Ref: 28) p. 498).

#### 26 **Selective Cleavage**

27 Selective cleavage of the polymer from the solid support can be carried out by a variety of  
28 methods: photolysis; catalytic hydrogenation; reaction with strong acids such as trifluoroace-  
29 tic acid, trifluoromethanesulfonic acid, hydrogen fluoride, and hydrogen bromide, preferably  
30 in the presence of a carbonium ion scavenger such as anisole or dimethyl sulfide; hydrolysis  
31 and alcoholysis catalyzed by nucleophiles such as ammonia, hydrazine, piperidine with dime-  
32

1 thylformamide, tributylphosphine with sodium fluoride; reductive cleavage of disulfide  
2 bonds; and enzymes. The choice of selective cleavage agent, besides depending on the amino  
3 acid composition of the peptide, must be compatible with the chemistry of the other groups,  
4 moieties, and/or molecules bound by covalent bonds to the peptide.

5 In a preferred embodiment in which a lanthanide macrocycle and a protein, such as an anti-  
6 body are linked to the polymer of the invention, both the lability of the macrocycle and the  
7 potential denaturation of the protein limit the choice of cleavage reagents. Strong chemicals,  
8 such as acids, bases, or any reaction involving organic solvents could cause either decomposi-  
9 tion of the macrocycle or denaturation of the protein.

10  
11 Two approaches to the cleavage of such peptide from the solid support are useful. One  
12 approach consists of attaching the peptide to the support via a disulfide linkage which can be  
13 cleaved by a reducing agent or by exchange with a sulfhydryl containing species, such as cys-  
14 teine and its derivatives(Ref. 26). A limitation to the use of this approach is the fact that the  
15 agents suitable for the cleavage reduction often also reduce disulfide bonds that either serve to  
16 attach an analyte-binding species to the polymer, or are essential to the structural integrity of  
17 proteins, such as antibodies, that constitute an analyte-binding species.

18 The second and preferred approach consists of including into the polymer an amino acid  
19 sequence that can be specifically cleaved by an enzyme at a rate significantly faster than the  
20 rate of destruction of the protein that constitutes the analyte-binding species. As will be  
21 described, Proteinase K, when combined with the appropriate amino acid sequence, is suffi-  
22 ciently selective to permit the safe recovery of polymers still attached to an antibody capable  
23 of binding to its antigen. If a protein other than this specific antibody is used, then an enzyme  
24 that has minimal effect on the protein and has a readily hydrolyzable peptide substrate is used.  
25 If the monomers are nucleotides, the substrate can be a sequence specific for a restriction  
26 endonuclease, such as the rare-cutters, BssHII from *Bacillus stearothermophilis* and NotI  
27 from *Nordcadia otitidis-caviarum* (Strachan and A. P. Read, 1999) (Ref. 29). An extensive  
28 description of synthetic nucleic acid chemistry and means to tag nucleotides is found in (Ref.  
29 26) Part III, 17. Nucleic acids pp 640-671, which is incorporated herein by reference.

30  
31  
32

## 1 Tags

2 Three types of tags are described in this invention: optical-labels, other-labels, and separa-  
3 tion-tags.

## 4 Optical-Labels

5 Three types of optical-labels are described in this invention: luminescence-labels, fluores-  
6 cence-labels, and absorbance-labels. It is highly desirable that species to be used as multiple  
7 luminescence-labels or fluorescence-labels should not suffer from concentration quenching.  
8 The best known examples of luminescence-labels that do not concentration quench are com-  
9 plexes containing lanthanide elements and having emission spectra with maxima in the range  
10 from 500 to 950 nanometers; such complexes consist of a trivalent lanthanide ion and an  
11 organic moiety.

12  
13 Lanthanide-containing Luminescence Labels. Particularly suitable luminescence-labels are  
14 the lanthanide-containing macrocycles, disclosed by L. Vallarino and R. Leif in US Patent  
15 5,696,240, whose entire disclosure is here incorporated by reference. The luminescence of the  
16 europium and samarium macrocycles can be enhanced by the addition of a solution which  
17 includes a nonluminescent trivalent lanthanide ion (Ref. 21).

18 Multiple M-Mac units linked to a polymer have the advantage of being insensitive to the  
19 concentration quenching that occurs with conventional organic fluorophores. Therefore, sig-  
20 nificant signal increase can be achieved by attaching a multiple M-Mac containing polymer to  
21 an analyte-binding species.

22  
23 The complexes of europium(III), dysprosium(III), samarium(III) and terbium(III), while  
24 not significantly luminescent in themselves, possess a long-lived luminescence in the pres-  
25 ence of an enhancer species. The enhancer species can be betadiketone molecules in solution,  
26 and can also be betadiketone groups present in the respective, europium, samarium, dyspro-  
27 sium, or terbium-tagged, polymers; in solution, these betadiketone molecules or betadiketo-  
28 nate groups are in equilibrium with the respective deprotonated species, namely the respective  
29 betadiketonate anions or betadiketonate groups. The intensity of europium(III), samarium(III)  
30 luminescence with a common enhancer species can be further increased by interaction with a  
31 cofluorescence solution. The samarium(III) macrocycle (SmMac) has been found to simulta-  
32 neously luminesce with the europium(III) macrocycle (EuMac) when a gadolinium(III)- or

1 yttrium(III)-containing cofluorescence solution essentially identical to the one previously  
2 described and containing 1,1,1-trifluoro-4(2-thienyl)-1,3-butanedione (HTTFA), (Ref. 21) is  
3 employed. It was also found that the aliphatic diketone, 1,1,1-trifluoro-5,5-dimethyl-2,4-hex-  
4 anedione (pivaloyltrifluoroacetone, HPTFA) interacts with both the terbium(III) macrocycle  
5 (TbMac) and the dysprosium(III) macrocycle (DyMac) to produce luminescent species.  
6 Therefore, it should now be possible to simultaneously and effectively employ four lumines-  
7 cent polymers, one labeled with a EuMac and emitting strongly in the red (ca. 618 nm), the  
8 second labeled with a SmMac and emitting in the orange and red at 564, 599, 645 and 652 nm,  
9 with the strongest SmMac emission occurring at 599 and 645-652 nm, the third labeled with a  
10 TbMac and emitting in the green (ca 545 nm), and the fourth labeled with DyMac and emit-  
11 ting in the blue and green at 480 and 575 nm.

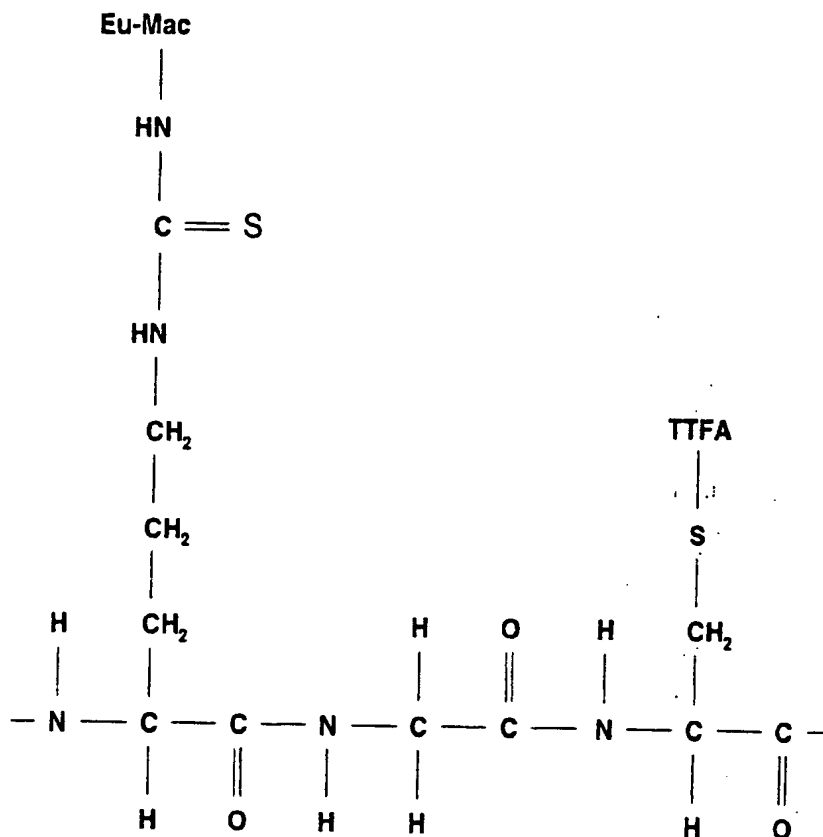
12 It is also possible to increase the number of available optical-labels by employing species  
13 containing the same set of fluorophores in different relative amounts, each mixed-fluorophore  
14 species serving as label for a given analyte, J. R. Kettman et al. 1998 (Ref. 30). A similar  
15 application is possible for the lanthanide macrocycles. Thus the narrow band emissions of lan-  
16 thanides make them excellent choices for use by themselves, or in combination with one  
17 another, or in combination with other luminescent or fluorescent optical-labels.  
18

19 According to this invention, the luminescent polymers tagged with Eu(III), Sm(III), Tb(III)  
20 and Dy(III) macrocycles can each be coupled to a different molecular species, which in turn is  
21 a member of a combining pair. In order to maximize the luminescence of each emitter -- the  
22 Eu(III), Sm(III), Tb(III) and Dy(III) macrocycles -- each emitter must interact with its optimal  
23 enhancing species. To this end, the solution containing the analytes to be detected/quantitated  
24 can be made up to include a common optimal enhancer for the EuMac and SmMac, for exam-  
25 ple HTTFA, and a separate optimal enhancer for the TbMac and DyMac, for example HPTFA.  
26 In such a situation, the luminescence of each of the lanthanides would be unavoidably  
27 decreased from its optimum level. Since the luminescence increasing ability of an enhancer  
28 depends on its electronic energy levels and is not related to a higher chemical affinity for the  
29 lanthanide it enhances, the probability that the EuMac, SmMac, TbMac, and DyMac would  
30 each have their two enhancer-binding positions occupied both by the appropriate enhancer  
31 would be reduced to about 25 percent in the presence of two "free" different enhancers in the  
32 common solution. It is also possible that an enhancer that is optimal for a first lanthanide ion

1 would accept energy from the enhancer for a second lanthanide ion resulting in a significant  
2 diminution of the emission from the second lanthanide ion.

3 Another approach to achieving optimized luminescence for the EuMac, SmMac, TbMac  
4 and DyMac is to bind at least one of the enhancers to the same polymer that includes the lan-  
5 thanide(III) macrocycle, in such a way that the geometric relationship between enhancer and  
6 lanthanide(III) macrocycle permits efficient energy transfer between the two species. For  
7 instance, multiple units of the betadiketone HPTFA, the anion of which (PTFA) preferentially  
8 enhances the luminescence of the TbMac and DyMac, can be bound to a polymer that con-  
9 tains multiple TbMac tags or multiple DyMac tags, whereas multiple units of the diketone  
10 HTTFA, the anion of which (TTFA) preferentially enhances the luminescence of the EuMac  
11 and SmMac, could be bound to a polymer that contains multiple EuMac tags or SmMac tags;  
12 both diketones being at all times in equilibrium with the respective anions. A peptide with a  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32

1 lanthanide-containing macrocycle and an enhancer attached to a neighboring amino acid  
 2 monomer unit is represented by the schematic Formula VI:



Formula VI

23       Formula VI is a schematic drawing of a peptide with a cationic Eu(III)-macrocylic moiety  
 24 (EuMac) bound by a thiourea linkage to a lysine and an anionic TTFA enhancer bound to the  
 25 sulfur atom of a cysteine. In Formula VI, the relative position of the EuMac and the TTFA  
 26 enhancer can be varied as required. Similar possibilities exist for PNAs and other lanthanide  
 27 complexes. The presence of these polymer-bound enhancers also serves to stabilize the lan-  
 28 thanide macrocycle complex by providing a suitably located counterion-ligand that is attached  
 29 to the same polymer chain.

30       Fluorescence-labels. Fluorescence-labels are most commonly large organic molecules with  
 31 double-bonded structures; they can be used singly or in combination to provide emission sig-  
 32

1 nals at different wavelengths. These fluorescence-labels usually have small Stokes shifts and  
2 their excitation and emission spectra partly overlap, resulting in the well-known phenomenon  
3 of fluorescence quenching when the individual absorbers/emitters are closely spaced on a  
4 polymer or other carrier. This effect, which is due to the transfer of energy between adjacent  
5 absorbers/emitters, can be minimized by designing and synthesizing peptides in which the flu-  
6 orescence-labels are spaced sufficiently apart, preferably by a distance within 5 to 50 Ang-  
7 stroms. There is a trade-off between the theoretical energy transfer efficiency, which is  
8 inversely proportional to the sixth power of the distance between the energy accepting and  
9 emitting species, Stryer and Haugland, 1967 (Ref. 31), and maximizing the number of fluores-  
10 cent optical-labels that can be attached to a peptide. However, it has been reported that this  
11 inverse sixth power relationship is not always observed, Y. Li and A. N. Glazer, 1999 (Ref.  
12 32). Thus, the optimum spacing between labels must be determined by experiment.

13 The preceding considerations also apply to appropriate combinations of organic fluoro-  
14 phores that can be specifically and sequentially linked to a peptide or other polymer according  
15 to the invention. With polymers containing multiple pairs of appropriately spaced different  
16 fluorophores, it becomes possible for a single light source, such as an Argon ion laser with a  
17 488 nm output, a mercury arc with a 365 nm output, or a HeNe or semiconductor laser, to  
18 excite two or more fluorophores with well separated excitation and emission spectra, but so  
19 selected that the emission spectrum of the "shorter-wavelength" fluorophore overlaps the  
20 excitation spectrum of the "longer wavelength" fluorophore. When the "shorter-wavelength"  
21 member of such an energy-transfer pair absorbs radiant energy and is excited, it transfers its  
22 energy through a nonradiative process to the "longer-wavelength" member, which is then  
23 excited and emits energy at its own characteristic wavelength. The close proximity of these  
24 energy-donor energy-transfer pairs maximizes the efficiency of energy transfer. Conversely,  
25 the separation of like fluorophores minimizes radiationless losses.  
26

27 Effective energy transfer between two or three appropriately positioned fluorophores can  
28 result in an increased separation between excitation and emission wavelengths, providing sets  
29 of fluorescent-labels that absorb at the same wavelength but emit at different wavelengths. For  
30 example, the following situation can exist: one label contains fluorophore A and produces A's  
31 typical emission. A second label contains fluorophores A and B; in this label, A absorbs light  
32 at its usual wavelength and transfers energy to B, which then emits light at considerably

1 longer wavelength than A itself. A third label contains fluorophores A, B and C, such that A  
2 absorbs light at its usual wavelength and transfers energy to B, which in turn transfers energy  
3 to C, and C finally emits light at longer wavelength than either A or B. Since the sequential  
4 synthesis of polymers from monomers with different side-chain reactive functionalities per-  
5 mits the manufacture of species with an effective spatial organization of light emitting and  
6 absorbing species, such sequential synthesis greatly improves the availability of fluorophore  
7 combinations capable of this energy absorbing/energy emitting cascade effect. The members  
8 of each energy-transfer set can be linked to monomer units located at specific positions along  
9 the polymer, in such a way that the distance between the members of each set, as well as their  
10 relative geometric orientations, provide efficient energy transfer between donor(s) and accep-  
11 tor(s) and minimize concentration quenching. Suitable energy transfer combinations include  
12 alpha-naphthyl groups and dansyl groups in the same molecule (Stryer and Haugland 1967),  
13 (Ref. 31), fluorescein and tetramethylrhodamine, 5-carboxyfluorescein and 5-carboxyX-  
14 rhodamine (Li and Glazer 1999), (Ref. 32), R-phycoerythrin (PE) and the cyanine dye Cy5™  
15 (Waggoner, et al. 1993), (Ref. 33), phycoerythrin-texas red (Ref. 34), phycoerythrin-cyanin  
16 5.1 (Ref. 34) and Peridinin-chlorophyll Rechtenwald, United States Patent No. 4,876,910,  
17 1989 (Ref. 35).

18 By taking advantage of the well-known secondary structures of peptides, such as alpha  
19 helices and beta pleated sheets, the distance and geometry between fluorescence-labeled  
20 monomers in peptides according to the invention can be computed and multiple polymers  
21 according to the invention can be synthesized with the technology of combinatorial chemistry;  
22 their fluorescence and/or luminescence spectra can serve as a screen to determine potential  
23 candidates for optical-labels in tagged-peptides created and used according to the teachings of  
24 this patent.

#### 25 Absorbance-Labels.

26 Tagged-polymer-analyte-binding species containing multiple absorbance-labels can be  
27 used in fields such as light microscopy and other analytical techniques, such as gel electro-  
28 phoresis. These tagged-polymer-analyte-binding species can replace the use of light absorbing  
29 enzyme products or light absorbing species produced by reactions involving enzyme products,  
30 and offer the advantage of employing a single antibody binding procedure, eliminating the  
31  
32



1 need for pretreating the sample to reduce background and of following the binding of the anti-  
2 body by an enzymatic development step. Tagged-polymer-analyte-binding species can elimi-  
3 nate the background absorbance associated with enzymatic reactions and provide selectivity  
4 of the light absorbing species with appropriate maxima, maximal extinction, and minimal  
5 spectral width. High absorbance intensity can be achieved by linking, according to this inven-  
6 tion, multiple chromophores to monomer units in a polymer. These chromophores can be con-  
7 ventional absorbance dyes or fluorescent species with a high molar absorbance, because  
8 fluorescence quenching has no significant effect on the increased absorbance provided by  
9 multiple light-absorbing moieties. An extensive description of light absorbing dyes of which  
10 many could serve as the basis of absorbance-labels is found in Gurr, 1971 (Ref. 36), which is  
11 incorporated herein by reference.

12 Other-Labels. Two types of other-labels are described in this invention: radioactive-labels  
13 and paramagnetic-labels. Radioactive-labels consist of any radioactive element or any ele-  
14 ment that can be induced to become radioactive and can be part of, or bound to, a monomer  
15 unit in the polymer of this invention. A particularly suitable radioactive-label is  $^{90}\text{Y}$  chelated  
16 to 1,4,7,10-tetraazacyclododecane- $\text{N,N',N'',N'''}$ -tetraacetic acid, DOTA, J. J. Peterson and C.  
17 F. Meares 1999 (Ref. 10). This radioactive-label can be formed by neutron bombardment from  
18 the nonradioactive Yttrium(III) macrocycle-monoisothiocyanate containing the isotope  $^{89}\text{Y}$ .  
19

20 Paramagnetic-labels. Paramagnetic-labels are species containing metal ions that have  
21 partly unfilled electron shells and hence possess permanent magnetic moments; certain para-  
22 magnetic labels can serve as contrast agents for magnetic resonance imaging. A paramagnetic  
23 label particularly suitable for this use is the gadolinium(III) macrocycle-mono-isothiocyanate.  
24 Polymers containing multiple gadolinium(III) complexes, which have high isotropic magnetic  
25 moments, can provide increased relaxivity for contrast enhancement in clinical magnetic reso-  
26 nance imaging (MRI). Attachment of a polymer carrying multiple gadolinium ions to a suit-  
27 able biomolecule further permits the targeting of the contrast agent to selected organs. The  
28 ordered synthesis of the polymers permits maximizing the localized gadolinium content while  
29 minimizing the general toxicity.

30  
31 Separation-tags. Four types of separation-tags are described in this invention: paramag-  
32 netic, charged, mass increasing, and density changing species; all these separation-tags

1 increase a specific physical property of the species to which they are bound. Thus, a molecule,  
2 particle, or cell bound to a polymer-analyte-binding species that is tagged with separation-  
3 labels will move under the appropriate force.

4     Paramagnetic separation-tags are species that contain highly paramagnetic metal ions. A  
5 molecule, particle, or cell attached to a polymer-analyte-binding species tagged with multiple  
6 paramagnetic separation-tags will migrate under a magnetic field gradient. Particularly useful  
7 for this purpose are the erbium(III) and holmium(III) macrocycle-mono-isothiocyanates.  
8

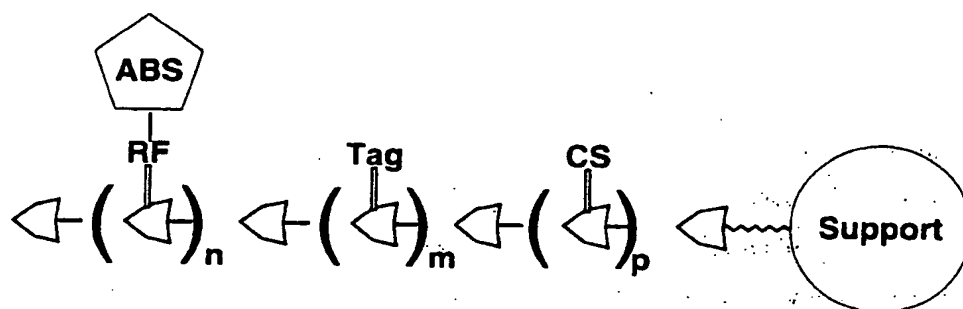
9     Charged-tags are species that contain highly charged metal ions. A molecule, particle or  
10 cell will change its net electrical charge after being attached to a polymer-analyte-binding spe-  
11 cies where the polymer includes multiple charged-tags. This will change both the electro-  
12 phoretic mobility and the isoelectric point of the molecule, particle or cell. Electrophoresis is a  
13 standard technique for separating molecules, particles, or cells under the effect of an electrical  
14 field. Each lanthanide(III) macrocycle adds a net charge of +3 to the polymer of a tagged-  
15 polymer-analyte-binding species and the bound molecule, particle, or cell.

16     Mass increasing tags and density changing tags are species that contain heavy metal ions;  
17 these tags increase the mass and the mass per unit volume, respectively, of any species to  
18 which they are attached. The increase in mass resulting from a mass-tag increases the  
19 response of the molecule, particle, or cell bound to a tagged-polymer-analyte-binding species  
20 to the application of a gravitational field, such as that induced by centrifugation. Similarly, a  
21 density-tag provides an increase in density. Gravitational fields are used to separate mole-  
22 cules, particles, and cells by both sedimentation velocity and buoyant density. Particularly  
23 useful for this purpose are the erbium(III) and holmium(III) macrocycle-mono-isothiocyan-  
24 ates.  
25

#### 26     **Analyte-Binding Species:**

27     There is also provided, according to this invention, a tagged-polymer-analyte-binding spe-  
28 cies comprising an analyte-binding species covalently attached to a tagged-polymer. Prepara-  
29 tion of this tagged-polymer-analyte-binding species is facilitated if the analyte-binding  
30 species reacts with the tagged-polymer while the latter is still attached to the solid support.  
31 This tagged-polymer-analyte-binding species can be represented by Formula VII, which  
32

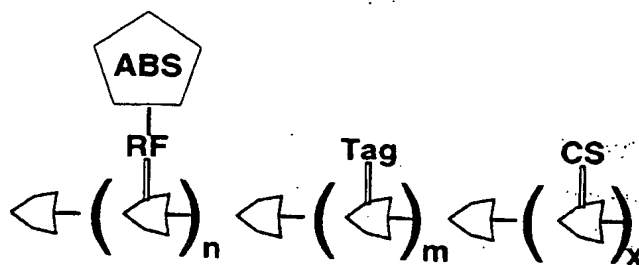
1 shows the analyte binding species, symbolized by ABS, linked to the polymer of the invention  
2 represented by Formula III above:



Formula VII

13 As in Formula III, each left pointing broad-arrow shape represents a monomer unit; RF  
14 represents a reactive functionality linked to a monomer unit; Tag independently at each occur-  
15 rence represents an optical-label, other-label, or separation-tag linked to a monomer unit; CS  
16 represents a cleavable link to the solid support shown by the circular shape at the right; the  
17 pentagon labeled ABS represents an analyte-binding species, linked by a covalent bond to a  
18 reactive functionality of the monomer and thus attached to the polymer. Broad-arrow shapes  
19 without other indication represent spacer monomer units: n, m, and p are numbers defined as  
20 above.

21 For certain uses, the tagged-polymer-analyte-binding species is freed from the support by  
22 selectively cleaving the cleavable link. The freed tagged-polymer-analyte-binding species can  
23 be represented by Formula VIII:



Formula VIII

1 in which, as in Formula VII, each left pointing broad-arrow shape represents a monomer  
2 unit; RF represents a reactive functionality linked to a monomer unit; Tag independently at  
3 each occurrence represents an optical-label, other-label, or separation-tag linked to a mono-  
4 mer unit; CS represents the monomer unit that was part of the cleavable link to the support; x  
5 is a number from 1 to 25 and is less than or equal to p of Formula VII; the pentagon labeled  
6 ABS represents an analyte-binding species, which has formed a covalent bond with a reactive  
7 functionality and thus is attached to the polymer; broad-arrow shapes without other indication  
8 represent spacer monomer units; and the numbers n and m are as defined above.

9  
10 The number of spacer monomer units such as spacer amino acids in a polypeptide accord-  
11 ing to the invention is governed by a balance of the cost of synthesis and the optimal number  
12 of spacers required to provide the desired three-dimensional conformation of the peptide. It  
13 can reasonably range from 0 to  $(20 \times m) + 100$ . Spacer monomers can be intercalated as appro-  
14 priate within or between groups of both tag-bearing and reactive functionality-bearing mono-  
15 mers. From 1 to 10 types of tags can be linked to monomer units.

16 When the polymer according to the invention is a peptide, the left pointing broad-arrow  
17 shapes with posts attached represent amino acids to which a tag or a reactive functionality can  
18 be linked, and the broad-arrow shapes without posts represent spacer amino acids. Non-limit-  
19 ing analyte-binding species include: proteins including antibodies, avidin and its derivatives  
20 and variants, streptavidin; nucleic acids and their analogs including DNA, RNA, and peptide  
21 nucleic acids (PNAs); lectins and analytes which are the tagged species in competitive assays.  
22 Many of the molecular genetic techniques required for the development and use of nucleic  
23 acid analyte-binding species are described in T. Strachan and A. P. Read, Human Molecular  
24 Genetics 2nd ed 1999 (Ref. 29).

25  
26 Before or subsequent to coupling to a protein, antibody, nucleic acid, other member of a  
27 specific combining pair, or extension to include a PNA, the polymer according to the inven-  
28 tion, such as a polypeptide, can be combined with any of the aforementioned species capable  
29 of forming a covalent bond with the reactive functionality of a monomer unit, such as a func-  
30 tionalized amino acid. For instance, lysine residues can react with the EuMac-mono-NCS.  
31 Cysteine residues, or other thiol-containing amino acids, can react with other thiols or with  
32 iodinated species of functionalized enhancers, March, 1985 (Ref. 37). Conversely, incorpora-

1 tion of an aliphatic iodine-bearing group in an amino acid can provide reactivity with thiols  
2 and other species. The result of each of the above-mentioned approaches will be a peptide  
3 containing luminescent or related molecules covalently bound in a specific order to some of  
4 its side chains. This peptide can be extended to form a PNA, or it can be terminated with a  
5 species which includes a reactive functionality capable of linking to a protein, nucleic acid,  
6 haptene or other relevant species employed in clinical assays. Both charged and uncharged  
7 naturally occurring or synthetic amino acids can be incorporated in the peptide for the pur-  
8 poses of increasing water solubility and minimizing nonspecific binding.

9 If the tagged-polymer, according to the invention, terminates in an oligonucleotide, this  
10 first oligonucleotide can be terminated by a sequence which is complementary to a region of a  
11 second oligonucleotide or polynucleotide. The two complementary regions of the first oligo-  
12 nucleotide and the second oligonucleotide or polynucleotide can hybridize. The first oligonu-  
13 cleotide can then be enzymatically extended in the presence of the 4 nucleotide triphosphates  
14 to form a region complementary to the second oligonucleotide or polynucleotide. This product  
15 after denaturation and separation from the second oligonucleotide or polynucleotide will be  
16 tagged-analyte-binding species that can be used to detect the sequences present in the second  
17 oligonucleotide or polynucleotide.

#### 18 19 Procedure

20 There is also provided, in accordance with this invention, a process for preparing a tagged  
21 water-soluble polymer comprising a plurality of tagged monomer units and spacer monomer  
22 units, with at least one of the tagged monomer units being tagged with an optical-label, or  
23 other-label, or separation-tag. The process consists of the following steps:

- 24 a) Providing a first monomer having 2-3 reactive functionalities, of which one is free  
25 and the remainder are protected,  
26
- 27 b) reacting the free reactive functionality of the first monomer with a water-insoluble  
28 support so as to link the monomer to the support,
- 29 c) deprotecting one protected reactive functionality of the monomer,  
30
- 31 d) providing a second monomer having 2-3 reactive functionalities, of which one is  
32 free and the remainder are protected; the first monomer and the second monomer can

- 1 be the same or different,
- 2 e) reacting the second monomer with the product of step c), thereby linking the second
- 3 monomer to the support through the first monomer,
- 4 f) deprotecting one remaining protected reactive functionality of the second monomer,
- 5 g) repeating steps d), e), and f) with additional monomers having 2-3 reactive
- 6 functionalities of which one is free and the remainder are protected, the additional
- 7 monomers being the same as, or different from, the first and/or second monomer,
- 8 thereby linking the additional monomers in predetermined number and sequence to
- 9 the support through the first monomer and the second monomer, to yield a polymer
- 10 comprising units of monomers in the number and sequence in which they have been
- 11 reacted and linked to the support,
- 12 h) as appropriate, sequentially or simultaneously deprotecting some or all of the
- 13 protected reactive functionalities,
- 14 i) as appropriate, sequentially or simultaneously reacting one or more tag(s), each
- 15 with a specific type of polymer-bound reactive functionality, to produce a tagged-
- 16 polymer
- 17 j) coupling an analyte-binding species to a specific type of peptide-bound reactive
- 18 functionality to produce a tagged-polymer-analyte-binding species
- 19 k) selectively cleaving the tagged-polymer-analyte-binding species from the support.
- 20
- 21

22 This sequence of steps produces a polymer that contains: at least one monomer unit linked  
23 to a tag including an optical-label capable of absorbing and/or emitting light at a wavelength  
24 between 200 and 1,400 nanometers, an other-label that is paramagnetic, or radioactive, or a  
25 separation tag that is a paramagnetic, or charged, or mass increasing, or density changing spe-  
26 cies; at least one monomer unit bearing a reactive functionality; and at least one spacer mono-  
27 mer unit. The molecular weight of the polymer of this invention is at least that of the essential  
28 three monomer units defined above. There is in principle no upper limit except the practical  
29 consideration that the added cost of more monomer units be justified by added benefits of  
30 their presence. Hence the polymer of the invention preferably includes from 3 to 1000 mono-  
31 mer units and more preferably has a molecular weight in the range from 1000 to 100000 dal-  
32 tons.

1 In this process of the invention, the tag can be an optical-label consisting of a macrocyclic  
2 complex of a lanthanide(III) ion. Particularly suitable are macrocyclic complexes in which the  
3 lanthanide ion is europium(III), samarium(III), dysprosium(III), or terbium(III).

4 The selective cleavage of the polymer from the support can be carried out by such mild  
5 techniques as enzymatic hydrolysis or disulfide reduction. In special cases, where both the tag  
6 and the analyte-binding species are both sufficiently resistant, cleavage can be achieved by  
7 photolysis, catalytic hydrogenation, or hydrolysis in presence of a nucleophilic catalyst or of a  
8 strong acid such as hydrofluoric acid or trifluoromethanesulfonic acid.  
9

10 When the monomers provided to the process are alpha-aminocarboxylic acids, the result-  
11 ing polymer is a tagged polypeptide bearing a reactive functionality for linking to an analyte-  
12 binding species.

13 Also in accordance with this invention, there is provided a method for the manufacture of  
14 the tagged-analyte-binding species. This method includes the steps of:  
15

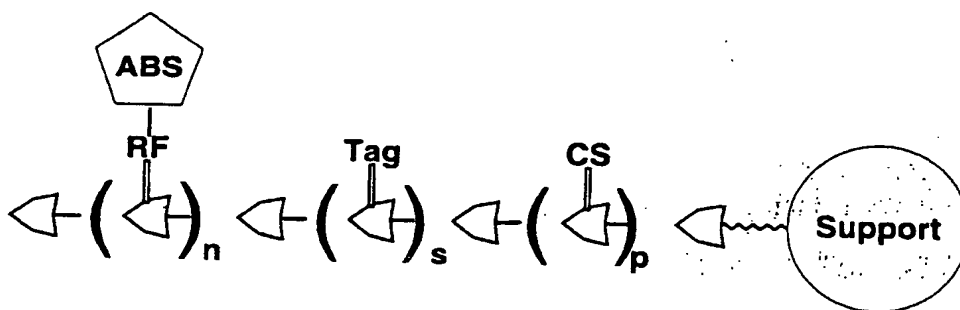
- 16 1. Producing a polymer that is bound to a solid support and contains three types of sites of  
17 known composition and sequence, as well as spacer monomer units. The first type of  
18 site includes either a sequence of monomers that can be specifically cleaved to permit  
19 the separation of the polymer from the solid support, or a single monomer that is cou-  
20 pled to the support by a bond that can be specifically cleaved. The second type of site  
21 includes either reactive functionalities or functionalities that include, are, or can be  
22 covalently coupled to, such tags as optical-labels, other-labels, or separation-tags. The  
23 third type of site has a specific reactive functionality capable of forming a covalent bond  
24 with an analyte-binding species. Additionally, the polymer can include spacer monomer  
25 units within and/or between these sites. The polymer contains at least one of each type  
26 of site and can contain more than one of each type, up to a practical upper limit where  
27 the added benefit of an additional site no longer justifies the effort of the added syn-  
28 thetic steps.
- 29 2. Deprotecting, if needed, specific reactive functionalities of monomer units in order to  
30 permit the coupling of tags to the monomer units by reaction with the deprotected reac-  
31

32

1. tive functionalities, if the tagged monomers are not already present because they were directly incorporated in the polymer.

3. Forming a specific covalent bond between a monomer unit with a reactive functionality and an analyte-binding species, such that number of monomer units so bonded equals the number of molecules of the analyte-binding species and is from 1 to 10 for each site of known composition and sequence.

8 Such a polymer can be represented by the schematic Formula IX:



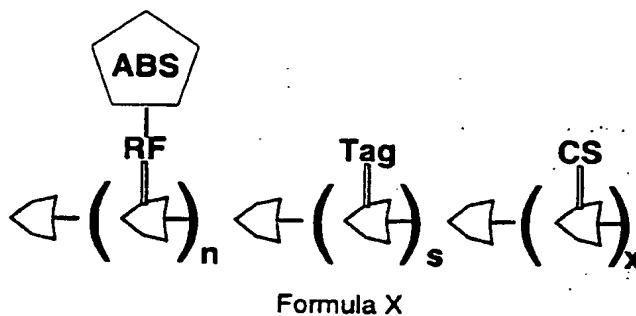
Formula IX

17 wherein each left pointing broad-arrow shape represents a monomer unit; RF represents a reactive functionality linked to a monomer unit; Tag independently at each occurrence represents an optical-label, other-label, or separation-tag linked to a monomer unit; CS represents a cleavable link to the solid support shown by the circular shape at the right; the pentagon labeled ABS represents an analyte-binding species linked to the polymer by a covalent bond to a monomer unit through a reactive functionality; broad-arrow shapes without other indication represent spacer monomer units, n is a number from 1 to 10, s is a number from 2 to 1000, and p is a number from 1 to 25.

4. Specifically cleaving the tagged-analyte-binding species from the solid support and releasing it into solution.

5. Such a polymer can be represented by the schematic Formula X:





9  
10 wherein, each left pointing broad-arrow shape represents a monomer unit; RF represents a  
11 reactive functionality linked to a monomer unit; Tag independently at each occurrence repre-  
12 sents an optical-label, other-label, or separation-tag linked to a monomer unit; the pentagon  
13 labeled ABS represents an analyte-binding species linked to the polymer by a covalent bond  
14 to a monomer unit through a reactive functionality; broad-arrow shapes without other indica-  
15 tion represent spacer monomer units, n is a number from 1 to 10, s is a number from 2 to 1000,  
16 and x is a number from 1 to 25 and is less than or equal to p of Formula IX.

17 The product of Step 2 can be stored as well as shipped. Hence, Steps 3 and 4 can be per-  
18 formed at any time after step 2, and the equipment and expertise required for steps 3 and 4 are  
19 much less than those required for steps 1 and 2. Accordingly, it is possible for a user to per-  
20 form these later steps conveniently at his/her own place of work, so that users dealing with  
21 many varieties of analyte-binding species will be able to label them and ship them on demand.

22  
23 The methodology of the present invention affords advantages compared to that of Peterson  
24 and Meares, 1998 (Ref. 9) because the purpose of the enzymatic cleavage of the peptide from  
25 the support is to manufacture a tagged-analyte-binding species used to produce an in vitro  
26 reagent. The reagent of this invention no longer includes the complete enzyme (Proteinase K)  
27 cleavable site. Peterson and Meares, 1999 only used the enzymatic cleavage (cathepsin B or  
28 cathepsin D) on the bead-bound peptides as a means to select sequences that would be cleaved  
29 in vivo. No enzymatic step was included in their preparation.

30 The methodology of the present invention offers advantages compared to that of Takalo et  
31 al. (Ref. 11) because: 1) the multiple fluorescing or luminescing containing moieties are cou-  
32 pled to a carrier; rather than being directly coupled to a biological molecule. The present

1 invention permits a large number of fluorescing or luminescing moieties to be attached with  
2 minimal loss of biological activity. 2) The chemical reactions employed for the attachment of  
3 the fluorescent or luminescent moieties are not limited to conditions that permit the retention  
4 of biological activity or the retention of the chemical integrity of the biomolecule.

5 The methodology of the present invention affords advantages compared to that of Kwiat-  
6 kowski et al.(Ref. 13) because the luminescent or fluorescent species that constitute the opti-  
7 cal-labels in the preferred embodiment are added after the peptide or polymer carrier has been  
8 synthesized on the solid support, and thus they are not subjected to the conditions required for  
9 any of the chemical reactions involved in the synthesis of the polymer.  
10

11 The methodology of the present invention affords advantages compared to those of both  
12 Takalo et al.(Ref. 11) and Kwiatkowski et al.(Ref. 13) because: 1) The tagged-polymer can be  
13 pre-manufactured and stored for subsequent use. 2) A biological analyte can be coupled to the  
14 tagged-polymer, containing luminescent or fluorescent optical-labels, under mild and/or phys-  
15 iological condition with minimal loss of biological activity. 4) The luminescent or fluorescent  
16 tagged biomolecule can be prepared for use with minimal equipment. and 5) The relative posi-  
17 tions of fluorescent or luminescent labeled groups can be controlled by the choice of their  
18 binding monomer, position, and intermediate spacer monomers. The well known ability of  
19 peptides to form secondary and tertiary structures can be employed to control the position and  
20 orientations of fluorescent and/or luminescent species.

21 The methodology of the present invention affords advantages compared to that of Salo et  
22 al. 1998 (Ref. 14) because 1) The tagged-polymer can be pre-manufactured and stored for  
23 subsequent use. 2) An oligonucleotide can be attached without the use of specialized, expen-  
24 sive instrumentation. 3) Enzymes can be used for selective cleavage of the polymer of the  
25 invention from the support. 4) A controlled geometry of the tagged monomer units can mini-  
26 mize radiationless losses between fluorescent species and between luminescent species with  
27 broad emissions. 5) A controlled geometry of the tagged monomer units can permit energy  
28 transfer between optical labels and 6) the polymer can be a peptide or PNA or any other spe-  
29 cies capable of sequential synthesis.  
30

31 Since multiple-optical-label polymers according to the invention provide greater signals  
32 than single optical-labels, they can be useful particularly as reporter molecules in immunoas-

1 says, analytical cytology, histological staining, and imaging processing. Multiple-optical-label  
2 polymers where the tag is a lanthanide macrocycle disclosed in US Patent 5,373,093 have the  
3 further advantages that the large Stokes shift, narrow band-width of the emission, enhance-  
4 ment of the emission by cofluorescence, and time gated luminescence minimize the back-  
5 ground noise. Thus, the signal can be maximized simultaneously with the noise being  
6 minimized. These luminescent polymers can be attached by a coupling functionality to small  
7 molecules, such as nucleic acid bases or haptens, or to large molecules like proteins, antibod-  
8 ies, or nucleic acids. These luminescent polymers can be linked to polynucleotides, peptide  
9 nucleic acids (PNAs), peptides, or polysaccharides.

10 Tags consisting of optical-labels, especially fluorophores, often require the presence of cer-  
11 tain proximal molecules or groups for efficient energy transfer and other purposes. The use of  
12 polymer carriers according to the invention permits different molecular species to be struc-  
13 tured in three-dimensional space to maximize the energy transfer from one optical-label to  
14 another. Luminescence enhancer species which absorb light and transfer energy to the lan-  
15 thanide can be located within the polymer structure in such a way that they can either complex  
16 directly with the lanthanide(III) ion of macrocyclic complexes, or transfer energy to an  
17 enhancer which is already directly complexed with the lanthanide(III) macrocycles. Thus, the  
18 sequential synthesis, according to the invention, of polymers from monomers with different  
19 side-chains permits the manufacture of species with an effective spatial organization of light  
20 emitting and light absorbing species.

21  
22 It is a feature of this invention that the polymers with functionalized side chains provide a  
23 means for attaching multiple luminescent lanthanide macrocycles to a single member of a  
24 combining pair or analyte-binding species resulting in increased signal; whereas the coupling  
25 to a polymer of multiple units of a conventional organic fluorophore, such as fluorescein, has  
26 not resulted in a significant increase in fluorescence compared to a single fluorophore unit.  
27 Therefore, the proportionality between luminescence intensity and macrocycle loading of a  
28 polymer, which is an essential aspect of the present disclosure, is not consistent with previous  
29 observations and hence is novel and unexpected.

30  
31 Three ways to covalently bind species with special desired properties (e.g luminescence) to  
32 a peptide backbone are: 1) Synthesize amino acids which have appropriately functionalized

1 and protected side chains and directly incorporate the species in the appropriate order as the  
2 peptide is synthesized. 2) Sequentially react a growing peptide, after the addition of a func-  
3 tionalized amino acid, with a species capable of forming a bond with the reactive functionality  
4 of said amino acid. The growing peptide presumably would be bound to a solid substrate. The  
5 species could be: an organic molecule (optical-label, luminescence enhancer, etc.), a metal ion  
6 containing macrocycle, or a chelate. 3) Include in the same peptide multiple amino acids with  
7 different reactive functionalities.

8 The luminescent polymers of the preferred embodiment of this invention are unique in sev-  
9 eral significant respects. The combination of properties which sets them apart from other fluo-  
10 rophores or fluorophore-binding polymers includes one or more of the following: a monotonic  
11 relationship between the number of luminescent species incorporated and luminescence inten-  
12 sity; reproducible, organized location of two or more molecular species capable of energy  
13 transfer from one species to another without direct covalent bonds between the species; solu-  
14 bility in aqueous solutions; controlled ionic charge and controlled hydrophobicity-hydrophi-  
15 licity to minimize nonspecific binding; and large Stokes shifts resulting from separation  
16 between excitation and emission spectra.  
17

18 Analytes linked to an analyte-binding species are conveniently grouped by molecular  
19 weights. One group of such analytes consists of compounds that have molecular weights in  
20 the range of about 125-2,000 daltons and include a wide variety of substances, which are often  
21 referred to as haptens. These compounds include:

- 22 a) Vitamins, vitamin precursors, and vitamin metabolites including retinol, vitamin  
23 K, cobalamin, biotin, folate;  
24  
25 b) Hormones and related compounds including  
26  
27 (i) steroid hormones including estrogen, corticosterone, testosterone, ecdysone,  
28  
29 (ii) aminoacid derived hormones including thyroxine, epinephrine,  
30  
31 (iii) prostaglandins,  
32  
31 (iv) peptide hormones including oxytocin, somatostatin,

- 1 c) pharmaceuticals including aspirin, penicillin, hydrochlorothiazide,  
2 d) Nucleic acid constituents including  
3  
4 (i) natural and synthetic nucleic acid bases including cytosine, thymine, adenine,  
5 guanine, uracil, derivatives of said bases including 5-bromouracil,  
6  
7 (ii) natural and synthetic nucleosides and deoxynucleosides including 2-deoxyad-  
8 enosine, 2-deoxycytidine, 2-deoxythymidine, 2-deoxyguanosine, 5-bromo-2-deox-  
9 yridine, adenosine, cytidine, uridine, guanosine, 5-bromo uridine,  
10  
11 (iii) natural and synthetic nucleotides including the mono, di, and triphosphates of  
12 2-deoxyadenosine, 2-deoxycytidine, 2-deoxythymidine, 2-deoxyguanosine, 5-bromo-  
13 2-deoxyuridine, adenosine, cytidine, uridine, guanosine, 5-bromouridine,  
14 e) drugs of abuse including cocaine, tetrahydrocannabinol,  
15 f) histological stains including fluorescein, DAPI  
16 g) pesticides including digitoxin,  
17 h) and miscellaneous haptens including diphenylhydantoin, quinidine, RDX.  
18 Another group of analytes consists of compounds having a molecular weight of 2,000 dal-  
19 tons or more; including  
20  
21 a) proteins and their combinations including  
22 (i) albumins, globulins, hemoglobin, staphylococcal protein A, alpha-fetoprotein,  
23 retinol-binding protein, avidin, streptavidin, C-reactive protein, collagen, keratin,  
24  
25 (ii) immunoglobulins including IgG, IgM, IgA, IgE,  
26  
27 (iii) hormones including lymphokines, follicle stimulating hormone, and thyroid  
28 stimulating hormone,  
29  
30 (iv) enzymes including trypsin, pepsin, reverse transcriptases  
31  
32 (v) cell surface antigens on T- and B-lymphocytes, i.e. CD-4, CD-8, CD-20 pro-  
teins, and the leukocyte cell surface antigens, such as described in the presently  
employed CD nomenclature;

- 1 (vi) blood group antigens including A, B and Rh,  
2  
3 (vii) major histocompatibility antigens both of class 1 and class 2,  
4  
5 (viii) hormone receptors including estrogen receptor, progesterone receptor, and  
6 glucocorticoid receptor,  
7  
8 (ix) cell cycle associated proteins including protein kinases, cyclins, PCNA, p53,  
9  
10 (x) antigens associated with cancer diagnosis and therapy including BRCA(s)  
11 carcinoembryonic antigen, HPV 16, HPV 18, MDR, c-neu; tumor suppressor proteins,  
12 p53 and retinoblastoma,  
13  
14 (xi) apoptosis related markers including annexin V, bak, bcl-2, fas caspases,  
15 nuclear matrix protein, cytochrome c, nucleosome,  
16  
17 b) toxins including cholera toxin, diphtheria toxin, and botulinum toxin, snake venom  
18 toxins, tetrodotoxin, saxitoxin,  
19  
20 c) lectins including concanavalin, wheat germ agglutinin, soy bean agglutinin,  
21  
22 d) polysialic acids including chitin;  
23  
24 e) polynucleotides including  
25  
26 (i) RNAs including segments of the HIV genome, human hemoglobin A and F  
27 messenger RNAs,  
28  
29 (ii) DNAs including chromosome specific sequences, centromeres, telomere spe-  
30 cific sequences, single copy sequences from normal tissues, single copy sequences  
31 from tumors.  
32

1 SUMMARY OF EQUIPMENT, INSTRUMENTS, GENERAL PROCEDURES AND  
2 MATERIALS

3  
4 Equipment, Instruments and General Procedures

5 In reporting quantities and concentrations, the term "micro" will be conventionally abbrevi-  
6 ated as u; for example, microgram will be abbreviated as ug.

7  
8 All glassware for EXAMPLE I to EXAMPLE III was cleaned with a methanol/concen-  
9 trated hydrochloric acid mixture (90/10 v/v), rinsed with deionized water and methanol, and  
10 dried at 60°C.

11 All aqueous solution of EXAMPLE I to EXAMPLE III were prepared using deionized  
12 water (Millipore, MilliQ Water, >18 MOhm resistance); Culligan distilled water 5 gallon is  
13 and was used in EXAMPLE IV onwards.

14  
15 Atomic absorption analyses of europium, samarium and terbium were performed on a  
16 Varian SpectraAA instrument, using as reference the elemental standards from Aldrich Chem-  
17 ical Co. (Eu, Catalog No. 20,712-8; Sm, 20,745-4; Tb, 30,592-8, 1996-97); selected samples  
18 were analyzed by ICP-AES (Schneider Laboratories, Richmond, VA).

19  
20 Fluorescence spectra of solutions were obtained with an SLM Model 8000 photon-count-  
21 ing spectrofluorometer. Samples were examined in stoppered triangular quartz cuvettes, so  
22 oriented that the excitation beam entered the diagonal face at a 45 degree angle and the emit-  
23 ted light was collected through the bulk of the sample at 90 degrees relative to excitation.

24 Visible/ultraviolet absorption spectra of solutions in EXAMPLE I to EXAMPLE III were  
25 obtained with a Shimadzu UV-265 ultraviolet-visible recording spectrophotometer, using  
26 stoppered quartz cuvettes. In EXAMPLE IV to EXAMPLE VII spectra were obtained with a  
27 Shimadzu UV 2401 PC model # 206-82301-92 spectrophotometer; samples were examined in  
28 stoppered 40 microliter quartz cuvettes (Starna, 16.40-Q-10).

29  
30 In experiments with peptide-bound PEGA beads (see next section); removal of supernatant  
31 was performed as follows: the PEGA beads with bound peptide were allowed to settle by  
32 gravity for approximately one minute prior to removing the supernatant fluid with a Gilson

1 Pipetman P200 and Fisher Brand 200uL pipetting tips (Fisher Scientific Catalog No. 21-197-  
2 2K). The fine bore of the pipetting tips prevented the entrance of the beads.

3 Eppendorf Safe-Lock 1.5 mL microcentrifuge tubes, Catalog Number 22 36 320-4  
4 (Eppendorf tubes) were used in all operations with the PEGA beads with bound peptide.  
5

6 All experiments and measurements were performed at ambient temperature unless stated  
7 otherwise.

8 All spectra were transferred to and graphed using a spreadsheet, Microsoft Excel.  
9

#### 10 MOST COMMONLY USED MATERIALS

11

12 (a) Hexamethylenetetramine (HMTA), ACS Reagent, Aldrich Chemical Co., Catalog  
13 No. 39,861-0 (1999).  
14

15 (b) Tris(hydroxymethyl)aminomethane (TRIS), ACS Reagent, Aldrich Chemical Co.,  
16 Catalog No. 25,285-9 (1996-97), (EXAMPLE I to EXAMPLE III). Examples IV onward,  
17 Ameresco Ultra Pure Grade, Catalog No. 0497-1Kg.

18 (c) Dimethylsulfoxide (DMSO), ACS Reagent, spectrophotometric grade, Aldrich  
19 Chemical Co., Catalog No. 15,493-9 (1996-97), (EXAMPLE I to EXAMPLE III).  
20

21 (d) EuMac-di-NCS, prepared according to procedures of Examples XI and XXXVI B,  
22 Step 1, of US Patent 5,696,240.

23 (e) 4,4,4-trifluoro-1(2-thienyl)-1,3-butanedione (thenoyltrifluoroacetone, HTTFA), Ald-  
24 rich Chemical Co., Catalog No. T2,700-6 (1996-97). For EXAMPLE I to EXAMPLE III,  
25 commercial HTTFA was purified by recrystallization from ethanol(charcoal)/hexane and  
26 stored at 4°C in a dark glass container. From EXAMPLE VII onwards, the HTTFA was used  
27 as received.  
28

29 (f) Aspartic acid, > 99%. SIGMA Catalog No. A8949 (1998)  
30

31 (g) Sephadex G-25 Superfine, Amersham Pharmacia, Code No. 17-0031-01 (1998-99).  
32



1 (h) High purity Gd(III) trichloride chloride hydrate (EXAMPLE I to EXAMPLE III),  
2  $\text{GdCl}_3 \cdot n(\text{H}_2\text{O})$ , prepared from the oxide,  $\text{Gd}_2\text{O}_3$  99.999% REO, Alfa Aesar, Catalog No.  
3 11289 (1999-2000), by dissolving it in 15% aqueous HCl, followed by evaporation to dryness  
4 with mild heating under reduced pressure. From EXAMPLE VII onwards,  $\text{GdCl}_3 \cdot 6\text{H}_2\text{O}$   
5 99.99%, Alfa Aesar, Catalog No. 11287 (1999).

6  
7 (i) 1,10-Phenanthroline (Phen), Aldrich Chemical Co., Catalog No.13,137-7 (1999).

8 (j) Cetyltrimethylammonium bromide (CTAB), Aldrich Chemical Co., Catalog  
9 No.85,582-0 (1999).

10  
11 (k) Trioctylphosphineoxide (TOPO), Aldrich Chemical Co., Catalog No.22,330-1  
12 (1999).

13 (l) The cofluorescence solution was prepared according to J.R. Quagliano et al: 2000  
14 (Ref. 21) (cofluorescence solution).

15  
16 (m) The hydrophilic support for peptide synthesis and manipulation was PL-PEGA  
17 Resin (Polymer Laboratories), which is described by the vendor as Acryloylated bis(2-amino-  
18 propyl)polyethylene glycol/dimethyl acrylamide copolymer, nominal particle size 300-500  
19  $\mu\text{m}$ , nominal loading 0.2mMol/g, abbreviated as PEGA.

20 (n) Proteinase K Molecular Biology, 23 mg/mL protein, 1,100 units, solution in 40%  
21 glycerol (v/v) containing 10 mM Tris-HCl, pH 7.5, with 1 mM calcium acetate, Sigma Cata-  
22 log. No. P-4850 (2000).

23  
24 (o) H-Cys(Npys)-Trp-Lys-Lys-Lys-Pro-Ala-Pro-Phe-Ala-Ala-Ala-LC-PEGA resin cus-  
25 tom synthesis, AnaSpec, Peptide Name: NIRL-2.

26  
27 Common inorganic acids, bases, and salts were obtained from ordinary commercial  
28 sources. Information for less commonly used materials will be provided in the Examples, as  
29 appropriate.

### 30 EXAMPLE I

#### 31 Synthesis of a Luminescent Lysine Homopolymer with Side Chains

32

1                    Consisting of a Hexa-aza-macrocyclic Complex of Europium(III)

2                    A. MATERIALS

3  
4            (a) EuMac-di-NCS (3.78 mg,  $4.0 \times 10^{-3}$  mol) dissolved in 0.900 mL of DMSO (EuMac-di-  
5 NCS DMSO solution).

6  
7            (b) HMTA aqueous solution (0.267 M) adjusted to pH 9.4 with NaOH (0.267 M HMTA pH  
8 9.4 buffer).

9            (c) Lysine homopolymer (5.1 mg,  $5.5 \times 10^{-5}$  mol) SIGMA Catalog No. P-1274, m.wt. 93,000  
10 dissolved in a mixture consisting of 0.400 mL DMSO and 1.00 mL 0.267 M HMTA pH 9.4. buffer  
11 (polylysine HMTA solution).

12  
13            (d) HTTFA ethanol solution ( $5.00 \times 10^{-2}$  M in ethanol-water), (HTTFA solution). The solution  
14 was prepared by dissolving 1.100 g of solid HTTFA in 5.00 mL of ethanol and diluting the result-  
15 ing solution to a total volume of 50.00 mL with deionized water. The solution was protected from  
16 light and stored in a refrigerator at 4°C.

17  
18            (e) HMTA, 10% aqueous solution (0.267 M), adjusted to pH 7.6 with hydrochloric acid,  
19 (0.267M HMTA pH 7.5 buffer).

20            (f) HMTA aqueous solution (0.71 M) adjusted to pH 6.0 with HCl, (0.71 M HMTA pH 6  
21 buffer).

22  
23            (g) Aspartic acid aqueous solution ( $2.0 \times 10^{-2}$  M), (aspartic acid solution).

24                    B. PROCEDURE

25  
26            (a) The EuMac-di-NCS DMSO solution (0.150 mL, 0.62 mg EuMac-di-NCS) was added  
27 with gentle shaking to a sample of polylysine HMTA solution. The mixture was allowed to stand at  
28 room temperature for 45 min, after which time 0.100 mL of  $2.0 \times 10^{-2}$  M aspartic acid was added  
29 with gentle shaking. The mixture was allowed to stand at room temperature for an additional 15  
30 min; it was then chromatographed through a column (17 cm height, 7 mm id) of Sephadex G-25 in  
31 0.267M HMTA pH 7.5 buffer. Elution with the same HMTA buffer, using a flow-cell detector (D-  
32 Star Instruments, DFW-20 Fixed Wavelength Detector) set for absorbance at 280 nm (absorption

1 of lysine-bound EuMac), gave the coupled peptide as a colorless solution. The eluate was  
2 divided into several portions. One portion was quantitatively analyzed for Eu by flame atomic  
3 absorption. Another portion was analyzed for polylysine by absorbance, using the Biuret tech-  
4 nique. (Dr. V. Katiyar/ [vishwa@alacran.metro.inter.edu](mailto:vishwa@alacran.metro.inter.edu)) The third portion was analyzed for  
5 Eu-luminescence as follows: 0.100 mL of eluate, 0.400 mL of  $5 \times 10^{-2}$  M HTTFA and 1.00 mL  
6 of a 0.71 M HMTA pH 6.0 were diluted with ethanol to 25.0 mL and the emission spectrum  
7 was obtained with excitation at 350 nm.

8  
9 (b) The procedure described in (a) was repeated using 5.3 mg of polylysine and 0.300  
10 mL of the EuMac-di-NCS DMSO solution (1.23 mg EuMac-di-NCS).

11 (c) The procedure described in (a) was repeated using 4.8 mg of polylysine and 0.470 mL  
12 of the EuMac-di-NCS DMSO solution (1.93 mg EuMac-di-NCS).

13  
14 The average yield of EuMac-coupled peptide in the three experiments was ca. 15% relative  
15 to the starting peptide. These experiments gave the following results, illustrated in Figure 1,  
16 Figure 2 and Figure 3: (1) The average EuMac-to-polylysine mole ratio in the coupled pep-  
17 tide, referred to as Eu-polylysine loading in the following, increased proportionally to the Eu/  
18 polylysine mole ratio used in the coupling reaction. (2) The emitted photon count, when nor-  
19 malized to account for different peptide concentrations, increased proportionally to the Eu-  
20 peptide loading. (3) The emission spectra of EuMac-polylysine samples with different Eu-

21

22

23

24

25

26

27

28

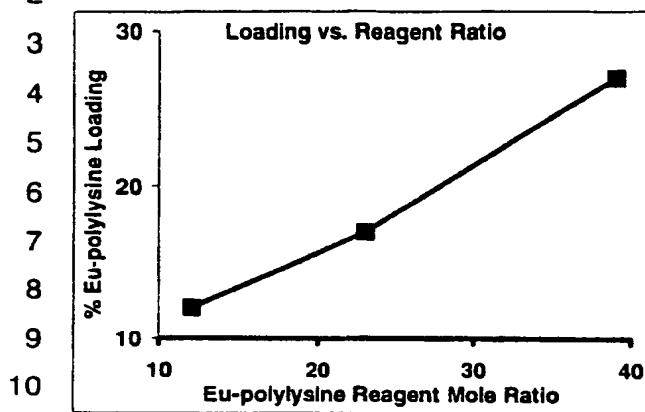
29

30

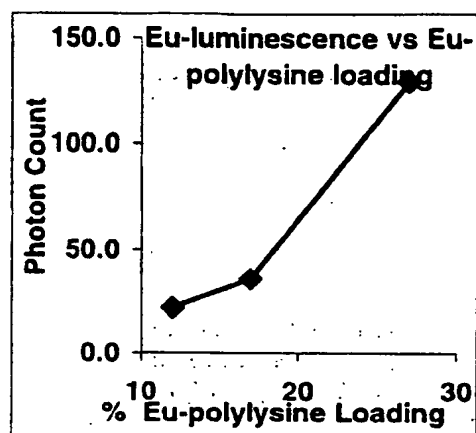
31

32

1 polylysine percentage loadings showed identical patterns, confirming that the emitting species  
2 are the same in each case.



12 Figure 1. Plot of the average EuMac-to-polylysine loading in coupled polylysine  
13 versus the EuMac-di-NCS-to-polylysine mole ratio used in the coupling reaction.  
14 The loading is expressed as percentage of  
15 EuMac-coupled lysine residues.  
16



29 Figure 2. Eu(III) emission at 618 nm nor-  
30 malized to  $1 \times 10^{-6}$  mmol polylysine /mL,  
31 as a function of Eu-polylysine percentage  
32 loading.

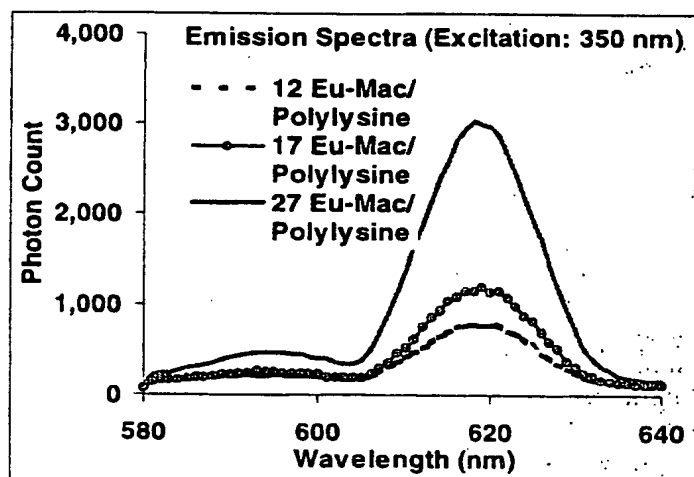


Figure 3. Eu-Emission spectra of EuMac-polylysine con-  
jugates at different Eu-polylysine percentage loadings.

## EXAMPLE II

Synthesis of a Luminescent Lysine-phenylalanine Copolymer with Side Chains Consisting of a Hexa-aza-macrocyclic Complex of Europium(III)

A. MATERIALS

(a) Lysine-phenylalanine (4:1) random copolymer (m.wt 47,200), Sigma Catalog No. P-3150 (Lysine-phenylalanine.)

(b) Other materials as in EXAMPLE I.

B. PROCEDURE

(a) The coupling and chromatography experiments described in EXAMPLE I were repeated using a lysine-phenylalanine (4:1) random copolymer, with the flow detector set for absorbance at 250 nm (phenylalanine absorption). The following quantities were used for the coupling reactions:

(i) Lysine-phenylalanine, 4.8 mg; EuMac-di-NCS, 0.566 mg; Eu/peptide reagents mole ratio = 5.32.

(ii) Lysine-phenylalanine, 5.3 mg; EuMac-di-NCS, 1.13 mg; Eu/peptide reagents mole ratio = 9.67.

(iii) Lysine-phenylalanine, 5.3 mg; EuMac-di-NCS, 1.81 mg; Eu/peptide reagents mole ratio = 15.5.

(b) The average yield of coupling-elution was ca. 18% relative to initial peptide. The eluates were analyzed for peptide using the BioRad technique (Bio-Rad Laboratories, Inc., US/EG Bulletin 1069), and for Eu-luminescence as described in EXAMPLE I. The results, summarized in Table 1 and Figure 4, showed that the normalized Eu-luminescence increased proportionally to the Eu/peptide reagent mole ratio.

**Table 1: Comparison of the emission intensities (as normalized photon counts) of EuMac-poly-lysine-phenylalanine copolymers obtained with different Eu-to-peptide reagent ratio**

Eu-Peptide Reagent Ratio	Normalized Photon Count
5.32	$4.69 \times 10^8$
9.67	$6.03 \times 10^8$
15.5	$7.9 \times 10^8$

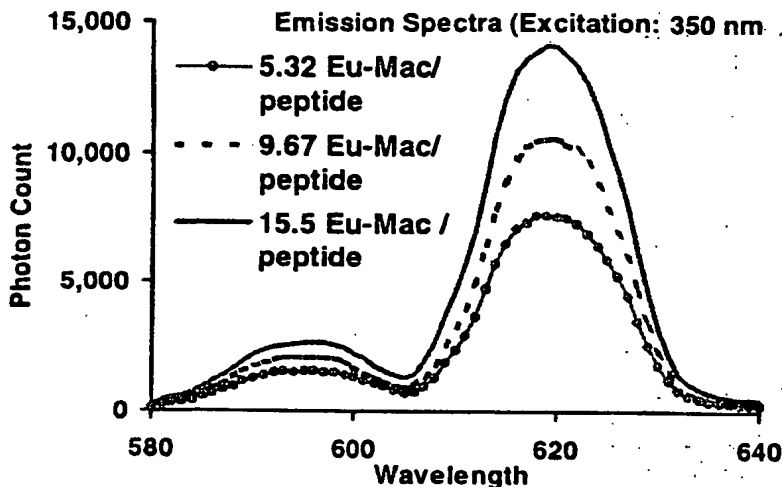


Figure 4. Eu-Emission spectra of EuMac-Polylysine-phenylalanine conjugates as a function of different Eu MacNCS/poly-lysine-phenylalanine ratios used in the coupling reactions.

### EXAMPLE III

#### Synthesis of a Luminescent Lysine-Tryptophan Copolymer with Side Chains Consisting of a Hexa-aza-macrocyclic Complex of Europium(III)

##### A. MATERIALS

(a) Lysine-tryptophan (4:1) random copolymer (m.wt. 38,000) Sigma Catalog No. P-9285, (Lysine-tryptophan).

(b) Other materials as in EXAMPLE I.

##### B. PROCEDURE

(a) The coupling and chromatography experiments described in EXAMPLE I were repeated using a lysine-tryptophan copolymer, with the flow detector set for absorbance at 280 nm (tryptophan and EuMac absorptions). The following quantities were used for the coupling reactions:

(i) Lysine-tryptophan, 4.9 mg; EuMac-di-NCS, 0.4 mg; Eu/peptide reagents

1 mole ratio = 3.26.

2 (ii) Lysine-tryptophan, 4.9 mg; EuMac-di-NCS, 0.8 mg; Eu/peptide reagents  
3 mole ratio = 6.51.  
4

5 **Table 2: Comparison of the emission intensities (as**  
6 **normalized photon counts) of EuMac-lysine-tryptophan**  
7 **copolymers obtained from different Eu-to-peptide reagent**  
8 **ratios.**

Eu-Peptide Reagent Ratio	Normalized Photon Count
3.26	$8.5 \times 10^7$
6.51	$34. \times 10^7$

A precipitate formed during the coupling reactions and the solutions were filtered prior to chromatography.

The average yield of Eu-coupled peptide was less than 10% relative to the initial peptide. The eluates

12 were analyzed for peptide by absorbance at 282 nm and for Eu-luminescence as described in  
13 EXAMPLE I. The results, summarized in Table 2, showed a regular increase in Eu-peptide  
14 loading with increasing Eu/peptide reagent mole ratio.  
15

16 The combined results of EXAMPLE I, EXAMPLE II, and EXAMPLE III clearly demon-  
17 strate that the polymer bound EuMac does not concentration quench and therefore the use of  
18 EuMac and other lanthanide optical-labels attached to a polymer is both scientifically and  
19 commercially feasible.  
20

#### 21 EXAMPLE IV

22 Selective Cleavage and Release (pH 7.1) of a Peptide Containing  
23 Amino Acids Capable of Forming Both Covalent Bonds with a  
24 Functionalized Dye and Forming Conjugates with a Member of a  
25 Specific Combining Pair

#### 26 A. MATERIALS:

27 (a) The Proteinase K cleavable peptide shown in Formula XI was synthesized on a Mer-  
28 rifield synthesizer by a commercial vendor, AnaSpec Inc. San Jose, CA (Peptide Name:  
29 NIRL-2) following standard commercial procedures, which are similar to those described by  
30 Peterson and Meares (Ref. 9). The carboxyl of the first amino acid, alanine, was covalently  
31 bonded to the amino functionalized version of the solid support, Polymer Laboratories, PL-  
32

1 PEGA Resin.

2 Formula XI shows a Proteinase K cleavable peptide bound to a PL-PEGA Resin bead. This  
3 structure shall be referred to as Peptide-PEGA-Bead(s). The peptide of Formula XI contains  
4 ProAlaProPhe(Ala)<sub>3</sub>, which is peptide VII of Table III of Bromme et al. 1986 (Ref. 42) Pep-  
5 tide VII has the highest ratio, 133,000 sec.<sup>-1</sup>mole<sup>-1</sup>, between the rate of catalysis and the  
6 Michaelis constant,. Bromme et al. (Ref. 42) describe this ratio as a measurement of protease  
7 activity.  
8



10  
11 Formula XI  
12

13 The Peptide of Formula XI includes 3 lysines, which can react with an isothiocyanate or  
14 other reactive functionality, such as those present on functionalized optical-labels. The 3-  
15 nitro-2-pyridinesulfonyl (NpyS) group is bound to the cysteine by a disulfide link which can  
16 subsequently undergo a disulfide exchange with an available cysteine or other sulfhydryl of an  
17 analyte-binding species, analyte, or member of a specific combining pair, such as an antibody.  
18 According to Menzo et al. 2000 (Ref. 43), The exchange with the antibody should be favored.

19 (b) Aqueous solution containing TRIS (0.01M) and CaCl<sub>2</sub> (0.001M) adjusted to pH 7.07  
20 with 10N and ca. 0.4N NaOH and with 12N and ca. 0.5N HCl, (Tris-Ca Buffer).  
21

22 (c) The Proteinase K was diluted 100th fold with distilled water to reach 230 ug/mL; 10  
23 uL of the 23 mg/ml stock solution with 990 uL of distilled water (Proteinase K).  
24

## 25 B. PROCEDURE

26 (a) The experiment, as described in Table 3, involved two samples: Control and 15.1 ug/  
27 mL of Proteinase K. The two samples of Peptide-PEGA-Beads were weighed in 1.5 mL  
28 Eppendorf tubes.  
29

30 (b) In order to maximize the sensitivity and precision of the measurement of the enzy-  
31 matic hydrolysis, the contamination by free peptide was minimized. The Peptide-PEGA-  
32 Beads were first washed by adding 200 uL of Tris-Ca Buffer, followed by vortex-mixing for



1 one minute (Wash 1). The Peptide-PEGA-Beads were allowed to settle by gravity and the  
2 supernatant was removed with a 200 uL tip Pipetman. A second 200 uL of Tris-Ca Buffer was  
3 added to the Peptide-PEGA-Beads, which were allowed to stay in the buffer 1.2 hours (Wash  
4 2). A 90 uL aliquot was removed from both washes with a 200 uL tip Pipetman. All opera-  
5 tions were performed at room temperature, approximately 25 °C.

6  
7 (c) 800 uL of Tris-Ca Buffer was added to the Peptide-PEGA-Beads, which were then  
8 vortex-mixed for a few seconds. The Peptide-PEGA-Beads were allowed to settle by gravity  
9 and subsequently a 90 uL aliquot was removed with a 200 uL tip Pipetman, (0 min. pre-addi-  
10 tion sample).

11 (d) The two washes and the 0 min. pre-addition sample from the Peptide-PEGA-Bead  
12 sample were subsequently transferred to 40 uL cuvettes and the absorbance spectrum was  
13 obtained with a spectrophotometer.

14  
15  
16 **Table 3. Enzymatic Hydrolysis Conditions**

17 <b>Experimental</b>	<b>Beads</b>	<b>Final</b>	<b>Buffer</b>	<b>Prot-K</b>	<b>Prot-K</b>	<b>dH<sub>2</sub>O</b>	<b>Total</b>
18 <b>Conditions</b>	<b>(mg)</b>	<b>Prot-K</b>	<b>(uL)</b>	<b>(ug)</b>	<b>stock</b>	<b>(uL)</b>	<b>Vol.</b>
19		<b>(ug/mL)</b>			<b>(uL)</b>		<b>(uL)</b>
20 <b>Control</b>	1.4	0	710	0	0	50	760
21 <b>15.1 Prot-K</b>	1.4	15.1	710	11.5	50	0	760

22  
23 (e) As shown in Figure 5, some of the peptide was washed off of the Peptide-PEGA-  
24 Beads prior to the addition of the Proteinase K. For both samples, the wash of the dry Peptide-  
25 PEGA-Beads, Wash 1, resulted in the largest loss of peptide (highest absorbance). The second  
26 wash, Wash 2, showed a smaller loss, and the 0 min pre-addition sample, which is equivalent  
27 to a third wash, showed an even lower loss.

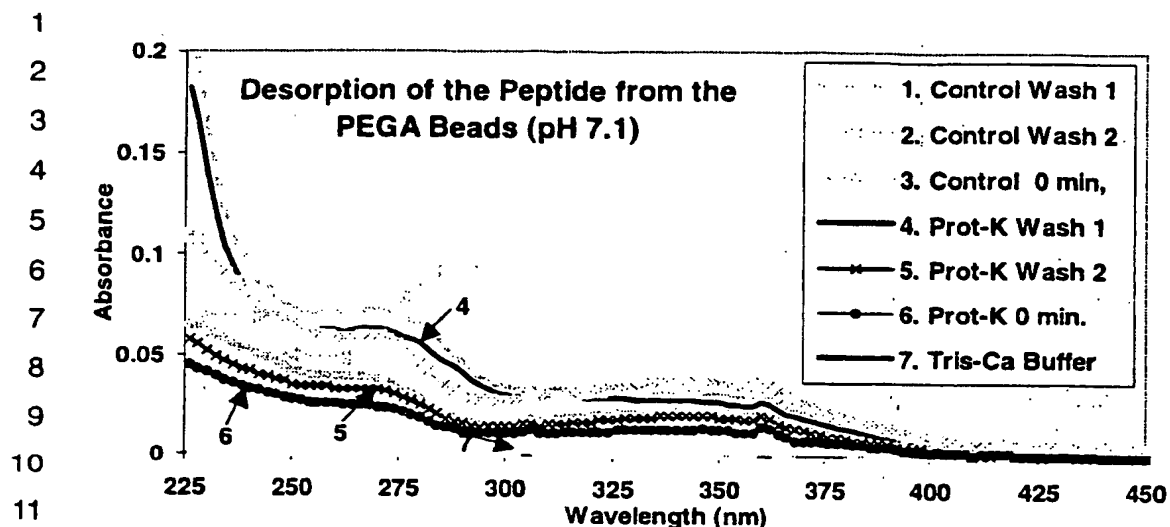


Figure 5. Desorption of the peptide of Formula XI from the Peptide-PEGA-Beads. The absorbance peaks at ca. 275 and at ca. 340 nm arise from the tryptophan residue and the NpyS, respectively. At this point in the experiment, which is prior to the addition of Proteinase K, both the Control and the Proteinase K samples are essentially identical except for a small difference in the amount of Peptide-PEGA-Beads

(f) Proteinase K (0 and 11.5 ug) was added to the two washed samples of Peptide-PEGA-beads, the total volume was brought up to 760 uL with Tris-Ca Buffer and water, and at selected times 90 uL aliquots of supernatant were obtained from the settled beads as described in (c).

(g) The aliquots of supernatant were transferred to a 40 uL cuvette and the absorbance spectra were obtained with a spectrophotometer.

(h) The data for the 0 min. pre-addition samples were also included as reference.

(i) After the addition of Proteinase K, the absorbance of the supernatants from both the Proteinase K sample and the Control sample increased above that of the respective 0-min pre-addition supernatants (Figure 6 and Figure 7). The supernatants from both the Proteinase K and the Control samples showed the 275 nm and 350 nm peaks characteristic of tryptophan and NpyS. However, the release of the free peptide was much greater for the Proteinase K sample.

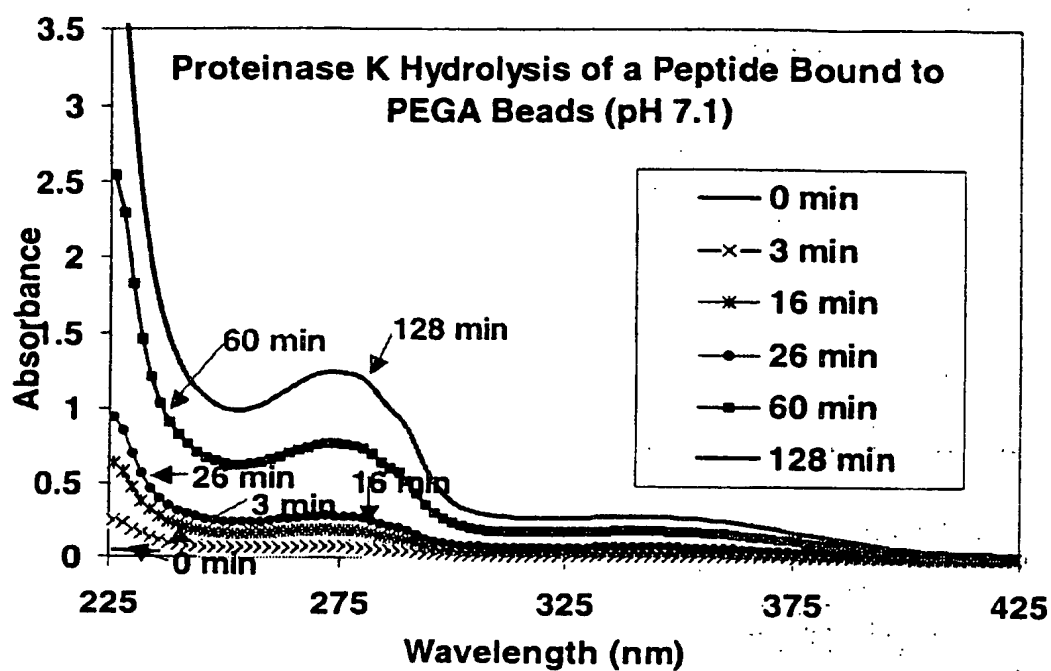


Figure 6. Proteinase K (15.1ug/mL) hydrolysis at pH 7.1 of the Peptide-PEGA-Beads (1.4 mg). The spectra indicates that, with time, Proteinase K cleaves the peptide from the solid support and that the released peptide includes both tryptophan and NpyS.

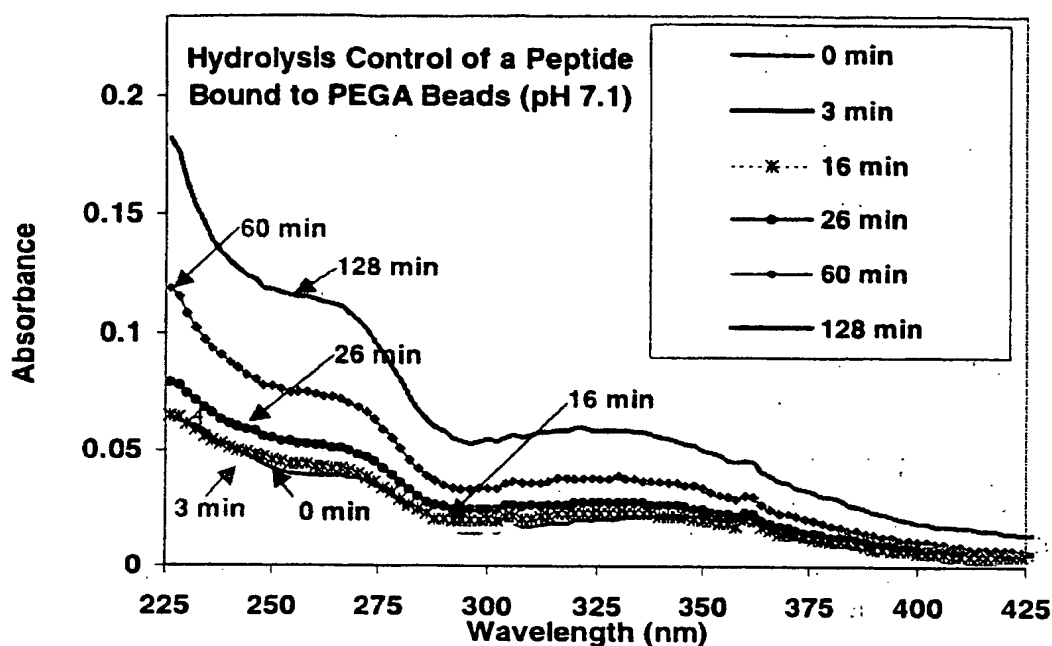


Figure 7. Hydrolysis (pH 7.1) of the Control sample of Peptide-PEGA-Beads (1.4mg). These spectra indicates that, with time, the peptide is slowly going into solution. Note that the ordinate scale is one fifteenth relative to that of Figure 6. No Proteinase K was present.

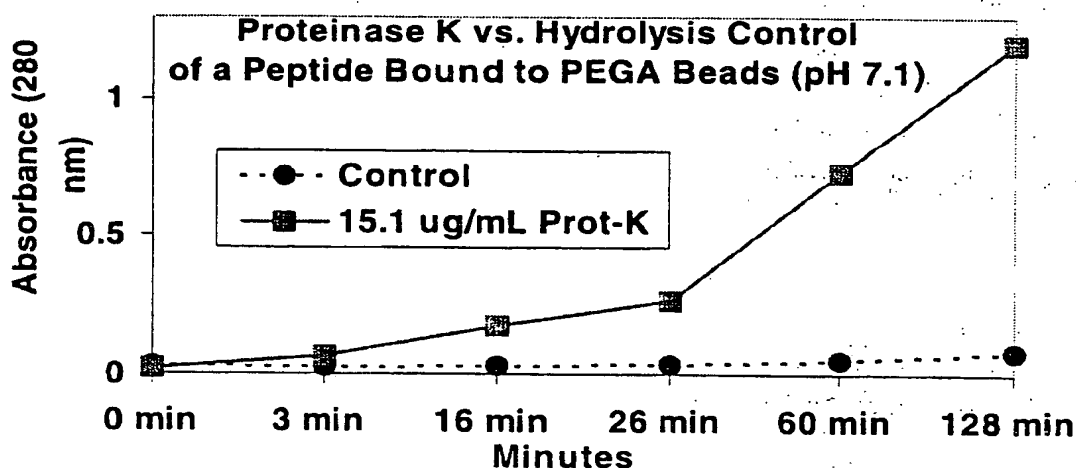


Figure 8. Graph of the absorbance of the supernatants for the Control and Proteinase K 15.1 ug/mL samples at 280 nm.

1 As shown in Figure 8, the increase in absorbance of the supernatants of the Control sample  
 2 was negligible compared to that of the supernatants of the Proteinase K sample. No apprecia-  
 3 ble amount of peptide was released spontaneously within the time required for significant  
 4 cleavage of the peptide by Proteinase K.

### 5 EXAMPLE V

#### 6 Selective Cleavage and Release (pH 8.0) of a Peptide Containing 7 Amino Acids Capable of Both Forming Covalent Bonds with a 8 Functionalized Dye and Forming Conjugates with a Member of a 9 Specific Combining Pair

#### 10 A. MATERIALS:

11 (a) The Tris-Ca Buffer of EXAMPLE IV adjusted to pH 8.01 with 10N and ca. 0.4N  
 12 NaOH and with 12N and ca. 0.5N HCl.

13 (b) All other materials as described in EXAMPLE IV.

14  
15  
16  
17  
18 **Table 4. Enzymatic Hydrolysis Conditions**

19 Experimental Conditions	20 Beads (mg)	21 Final Prot-K (ug/mL)	22 Buffer (uL)	23 Prot-K (ug)	24 Prot-K stock (uL)	25 H <sub>2</sub> O (uL)	26 Final Vol. (uL)
27 Control	1.2	0	710	0	0	100	810
28 14.2 Prot-K	1.1	14.2	710	11.5	50	50	810
29 28.4 Prot-K	1.1	28.4	710	23	100	0	810

#### 30 B. PROCEDURE:

31 (a) The experiment, as summarized in Table 4, involved three samples: Control, 14.2 ug/  
 32 mL Proteinase K and 28.4 ug/mL Proteinase K. The Peptide-PEGA-Beads were weighed in  
 33 1.5 mL Eppendorf tubes.

(b) The procedures of EXAMPLE IV were followed, with the exceptions that the hydrol-

1 ysis was carried out at pH 8.01, two concentrations of Proteinase K were studied, and Wash 1  
2 and Wash 2 were combined.

3 (c) After the addition of 14.2 ug/mL of Proteinase K, the absorbance of the supernatants  
4 increased with time as illustrated in Figure 9. Similar spectra (not shown) were obtained for  
5 the sample treated with 28.4 ug of Proteinase K. Both the spectra at 156 min, (Figure 10) and  
6 the change of absorbance with time (Figure 11) demonstrate that Proteinase K cleaves a pep-  
7 tide from the Peptide-PEGA-Beads. The concentration of the peptide thus cleaved is much  
8 greater than that present in the supernatant of the Control sample or in any of the three Com-  
9 bined Washes. The doubling of the enzyme concentration resulted in an approximately 1.3  
10 fold increase in cleaved peptide (Figure 11).  
11

12 Both the supernatants from the Proteinase K samples and the Control sample showed the  
13 275 nm peak due to tryptophan absorption (Figure 10). However, the 350 nm peak from the  
14 NpyS that was observed at pH 7.1 is no longer discernible.

15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32

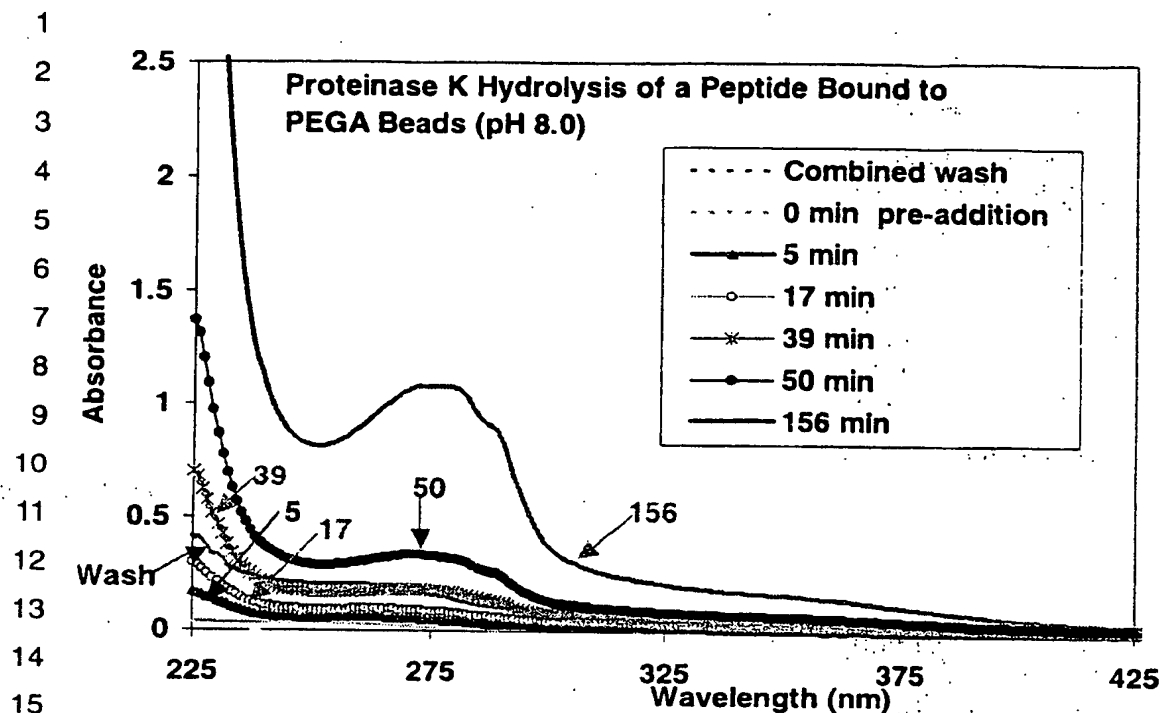
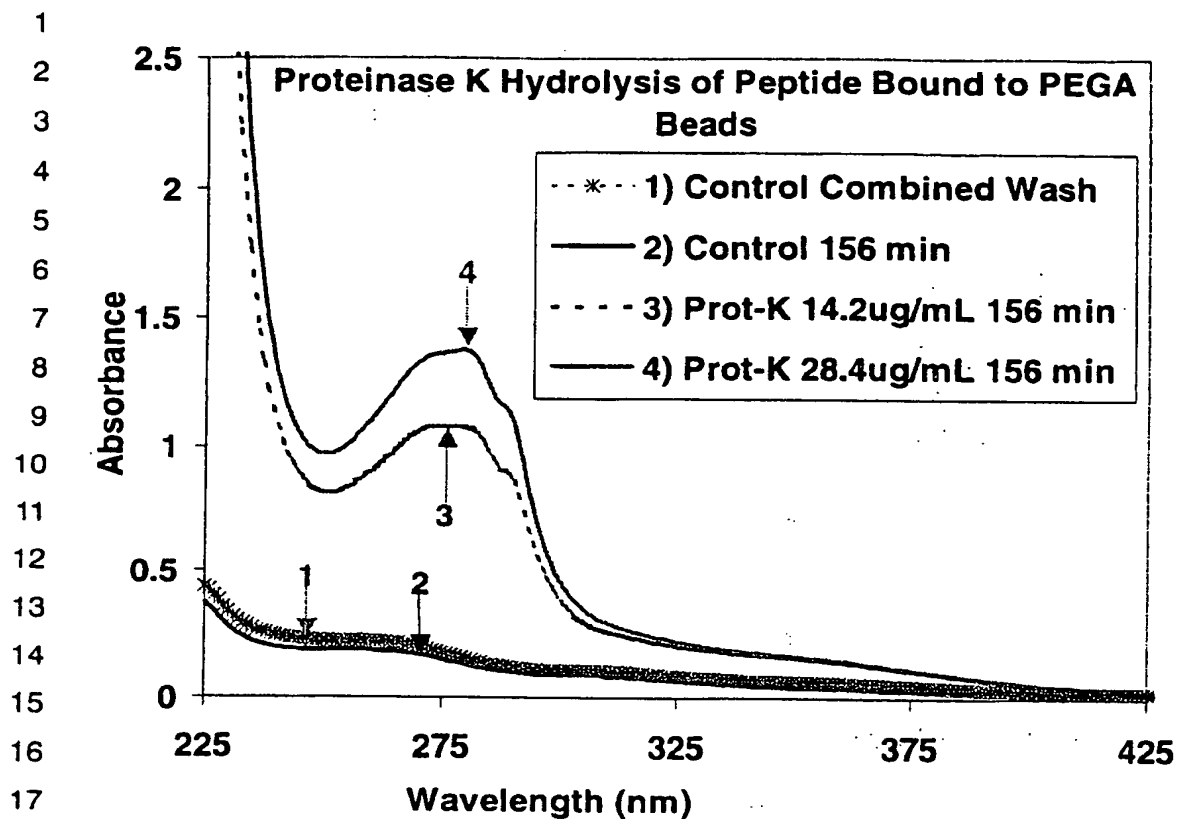


Figure 9. Proteinase K (14.2 $\mu$ g/mL) hydrolysis at pH 8.01 of the Peptide-PEGA-Beads (1.1mg). The spectra indicate that with time (5 to 156 min), Proteinase K cleaves the peptide, and that the released peptide includes both tryptophan and NpyS. A small amount of the Peptide bound to the Peptide-PEGA-Beads is initially washed off (Combined Wash). The preaddition, 0-min sample shows minimal background. At pH 8 the NpyS absorbance at 350nm is reduced.



18 Figure 10. The Combined Washes of the two Proteinase K samples (not shown) were  
19 essentially the same as the one from the Control. The spectra indicate that, after 156  
20 min, both concentrations of Proteinase K cleave the peptide from the solid support. All  
21 four spectra show a clear tryptophan peak at 275-280nm.

22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32



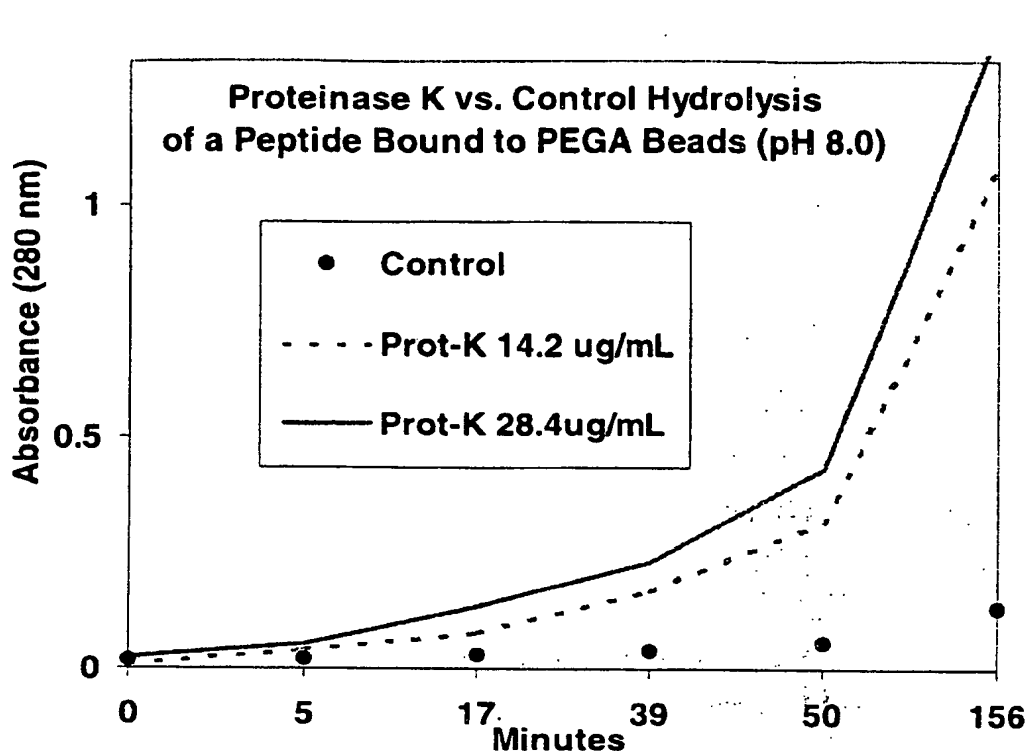


Figure 11. The graph shows how the absorbance at 280 nm of the supernatants from the Control sample and of the Proteinase K 14.2 and 28.4 ug/mL samples increases with time. The ratio of released peptide for the 28.4 vs. 14.2 ug/mL is approximately 1.3.

#### EXAMPLE VI

##### Demonstration of the Resistance of a Monoclonal Antibody to Proteinase K Digestion

#### A. MATERIALS

(a) The Tris-Ca Buffer of EXAMPLE V adjusted to pH 8.06.

(b) PRB-1, an antibody specific for the 5BrdU marker for DNA (Anti5BrdU) and labeled with a fluorescein analog, available from Phoenix Flow Systems, Catalog No. ABFM18, San Diego, California.

(c) The containers used for the experiment were Fisher 5 mL polystyrene round bottom

1 tubes, 12 x 75 mm style, Fisher Scientific Catalog No. 2008.

2 (d) The reagents in the Phoenix Flow APO-BRDU kit, Catalog No: Au1001, were used  
3 for the measurements.  
4

#### 5 B. PROCEDURE

6 The resistance of labeled Anti5BrdU to Proteinase K was demonstrated by the use of this  
7 antibody in the measurement of apoptosis, employing commercial flow cytometry reagents  
8 and procedures. A comparison was made between the antibody before and after enzymatic  
9 digestion with two concentrations of Proteinase K. Apoptosis results in DNA strand breaks  
10 terminated by 3'-hydroxyl ends. In the absence of a template, terminal deoxytransferase, TdT,  
11 adds nucleotides to these ends including the analog BrdUTP. The Phoenix Flow kit included  
12 an anti-BrUdR coupled to a 488nm excited fluorescent dye. The anti-BrUdR binds to the  
13 incorporated BrdU. The Propidium iodide/RNase solution from the APO-BRDU kit was used  
14 according to the manufacturer's instructions to specifically stain the total DNA.  
15

16 (a) Proteinase K was diluted with the pH 8.08 Tris-Ca Buffer to concentrations of 24 and  
17 240 ug/mL. The Anti-5BrUdR was diluted to 0.1 ug/uL with the pH 8.01 Tris-Ca Buffer,  
18 according to the published instructions. Proteinase K was added and the samples were incu-  
19 bated at room temperature for 58 min.

20  
21 (b) Within less than 5 minutes after the end of the incubation, one mL,  $1.0 \times 10^6$  cells, of  
22 previously prepared BrdUrD labeled control cells were added to a mixture of 90 uL of Rinse  
23 Buffer of the Phoenix Flow kit and 10 uL of the Anti5BrdU solution. The cells with the  
24 labeled Anti5BrdU solution were incubated in the dark for 30 minutes at room temperature.  
25 0.5 mL of the Propidium Iodide/RNase A Solution was added to stain the DNA. The 5mL  
26 tubes were wrapped with aluminum foil and the cells were incubated in the dark for 30 min-  
27 utes at room temperature.

28 (c) After incubation, a FACScan (Becton Dickenson) flow cytometer equipped with a  
29 488nm laser and logarithmic amplifiers was used to measure the cells fluorescence arising  
30 from both the fluorescein analog labeled Anti5BrdU and the Propidium Iodide.  
31

32 (d) The results shown in Table 5 demonstrated that 24 ug/mL of Proteinase K had negli-

1 gible effect on the antibody and that even after exposure to a 10 times greater Proteinase K  
2 concentration, 65% of the positive cells could still be detected. For the 0 (control), 24, and 240  
3 ug/mL Proteinase K treatments, the differences between the positive and negative channels  
4 were 440, 432, and 289 respectively. Note that a concentration of 24 ug/mL is approximately  
5 equal to the 28.4 ug/mL concentration and twice the 14.2 ug/mL concentration used in  
6 EXAMPLE V (Table 4). Thus, a significant amount of biologically active antibody survived  
7 the enzymatic hydrolysis condition of the peptide of EXAMPLE IV and EXAMPLE V.

8  
9  
10 **Table 5. Effect of Proteinase K Treatment on Anti5BrdU**

11 Prot-K (ug/mL)	12 Anti- 5BrdU ug/uL	13 % Fluor. Cells	14 Mean Channel of Pos. Cells	15 Mean Channel of Neg. Cells	16 Pos. - Neg. Mean Channel
17 0	0.1	36.7	675	235	440
18 24	0.1	35.5	681	249	432
19 240	0.1	24.0	524	235	289

EXAMPLE VII

Coupling of a Functionalized Europium Macrocycle to the PEGA Bound  
Peptide of EXAMPLE IV and Release of the Europium Macrocycle  
Labeled Peptide by Enzymatic Hydrolysis

A. MATERIALS

- (a) Dimethylsulfoxide (DMSO) ACS Reagent, Sigma Catalog No. D-8779.
- (b) EuMac-mono-NCS in DMSO solution ( $5.4 \times 10^{-3}$  M, 4.6mg/mL).
- (c) The Peptide-PEGA-Beads, Formula XI of EXAMPLE IV A (a).
- (d) HMTA 0.267 M solution in water, adjusted to pH 9.45 with NaOH (0.267 M HMTA pH 9.45 buffer).
- (e) HMTA 0.267 M solution in water, adjusted to pH 7.5 with HCl (0.267 M HMTA pH 7.55 buffer).
- (f) Tris-Ca Buffer adjusted to pH 8.0 with 1M HCl (Tris-Ca pH 8.0 buffer).
- (g) Proteinase K 0.46 ug/uL in Tris-Ca pH 8.06 buffer (Proteinase K solution).
- (h) Cofluorescence solution prepared with  $\text{GdCl}_3 \cdot 6\text{H}_2\text{O}$  (99.99%); Alfa Aesar, Catalog No. 11287 (1999).

B. PROCEDURE

- (a) 2.3 mg of the Peptide-PEGA-Beads were weighed in a 1.5 mL Eppendorf tube.
- (b) A mixture consisting of 0.20 mL of DMSO and 0.50 mL of the 0.267 M HMTA pH 9.45 buffer was added to the Peptide-PEGA-Beads, which were then dispersed by Vortex-mixing for approximately 2 minutes. The EuMac-mono-NCS solution (0.150 mL, 0.69 mg EuMac-mono-NCS) was slowly added with gentle tapping to suspend the Peptide-PEGA-Beads. The total volume was 850 uL.
- (c) The Peptide-PEGA-Beads were allowed to stand at room temperature for 45 min and

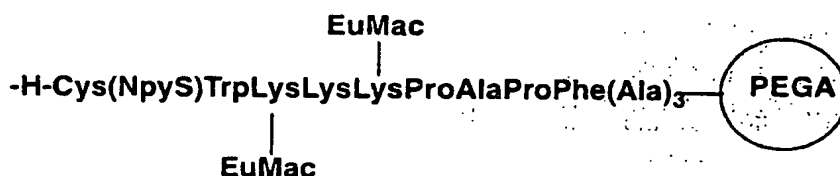
1 allowed to settle by gravity. Subsequently the buffer was removed with a 200uL tip Pipetman.

2 (d) Step (b) was repeated.

3  
4 (e) Step (c) was repeated except that the Peptide-PEGA-Beads were allowed to stand at  
5 room temperature for 53 min.

6  
7 (f) The Peptide-PEGA-Beads were then washed four times with 150 uL of HMTA pH  
8 7.55 buffer. This washing restored the EuMac to neutrality and removed contaminants, such  
9 as any unbound EuMac-mono-NCS. Formula XII shows the Peptide-PEGA-Beads with  
10 EuMac bound to the lysine residues. The position and number of the EuMac in Formula XII is  
11 diagrammatic. The number of EuMacs bound on each peptide ranged from 0 up to 3. This  
12 structure shall be referred to as EuMac-Peptide-PEGA beads.

13 (g) The EuMac-Peptide-PEGA-Beads can be stored at this time in either dimethylforma-  
14 mide or ethanol at -20°C or below.



22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32

Formula XII

23 (h) The Peptide-PEGA-Beads were then washed two times with 150 uL of Tris-Ca pH  
24 8.0 buffer and finally suspended with 426 uL of Tris-Ca pH 8.0 buffer.

25 (i) The Proteinase K solution (25 uL, 11.5 ug) was then added to the EuMac-Peptide-  
26 PEGA-Beads resulting in a total volume of 451 uL and a Proteinase K concentration of 27.0  
27 ug/mL.

28  
29 (j) The EuMac-Peptide-PEGA-Beads were allowed to settle for approximately one  
30 minute, 70 ul aliquots of the supernatant were removed at 40, 80, 115, and 124 minutes using  
31 a 200 uL tip Pipetman, and the absorbance spectra were obtained with a spectrophotometer  
32 employing 40 uL cuvettes.

(k) The increasing absorbance readings at 280 nm, shown in Figure 12, indicate that the Proteinase K did release a cleavage product containing tryptophan from the EuMac-Peptide-PEGA-Beads.

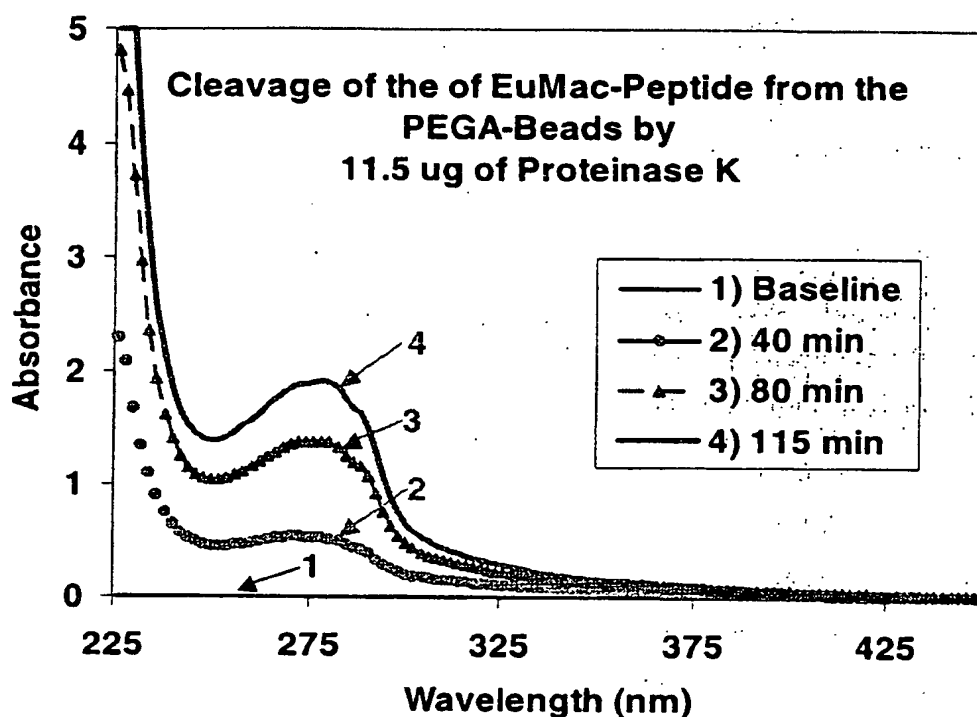


Figure 12. Graph of supernatant from Proteinase K hydrolysis of EuMac-(mono)-NCS and NIRL-2 beads conjugate. The graph shows that the absorbance at about 280 nm increases with time for all samples. The longer the hydrolysis time, the higher the absorbance results at about 280 nm. This demonstrates that Proteinase K cleaves the peptide from the PEGA-Beads.

(l) All EuMac-Peptide-PEGA-Bead samples were stored at 8° C. Small samples of beads were mixed with the cofluorescence solution for observation with an episcopic fluorescence microscope equipped with a 10X objective 0.25 N.A. The UV illumination was provided by a 100 watt Mercury-Xenon short arc. The fluorescence was excited at 365 nm and the emitted light was observed through an Omega Optical Ploemopak cube, UV DAPI, equipped with the following: a 365 nm narrow-band-width excitation filter (Omega 365HT25), a 400 nm Beam-

1 splitter (Omega 400DCLP02), and a two-band 450 and above 600 nm emission filter (Omega  
2 450DF65). The CCD optical path was equipped with a 619 nm narrow-band, 5.6 half-width,  
3 emission filter (Omega 618.6NB5.6). The images were obtained with an uncooled EDC-  
4 1000N CCD camera (652 x 494). The gray levels of the images were inverted for display.  
5 Darkness indicates strong luminescence.

6 Both the pre-hydrolysis sample of the EuMac-Peptide-PEGA-Beads and the sample hydro-  
7 lyzed for 115 min fluoresced under UV excitation (Figure 13). However, the luminescence  
8 from the pre-hydrolysis sample was strong and the luminescence from the sample hydrolyzed  
9 for 115 min was weak. The strong luminescence demonstrated that significant amount of  
10 EuMac had coupled to the peptide. The drastic difference in luminescence before and after  
11 Proteinase K hydrolysis demonstrated that the EuMac-labeled part of the peptide was released  
12 from the bead.  
13

14 The periphery of the pre-hydrolysis sample bead also has a luminescence, but this was not  
15 as bright as that of the bead itself (Figure 13 Left). A reasonable explanation for this lumines-  
16 cence "halo" from the solution immediately surrounding the pre-hydrolysis bead is that it  
17 results from the EuMac-Peptide attached to the polyethylene glycol pendant polymer side  
18 chains that emanate from the PEGA-BEAD. The amount of EuMac-Peptide contained in this  
19 halo could have been considerable because the image observed through a microscope is a two-  
20 dimensional section of a three-dimensional object. To test for luminescence in the supernatant,  
21 a spot-test was performed by placing 2 uL of the hydrolyzed supernatant sample (115 minutes  
22 into the hydrolysis) on a slide with 2 uL of the cofluorescence solution; the spot did luminesce  
23 when irradiated at approximately 365 nm.

24 Combining the results of EXAMPLE IV to EXAMPLE VII proves that it is feasible to pre-  
25 pare peptides with an enzyme-cleavable site, conjugate the peptide with an optical-tag; in this  
26 case a lanthanide(III) macrocycle, and to enzymatically cleave the conjugated peptide from its  
27 support under conditions that do not significantly reduce the activity of an analyte-binding  
28 species, in this case an antibody.  
29

30  
31  
32



Figure 13. CCD Images (619 nm emission) of a EuMac-Peptide-PEGA-Beads with added cofluorescence solution, before hydrolysis with Proteinase K (Left) and after 115 minutes of hydrolysis (Right). The exposure was 500 ms with a 10X, N.A 0.25 objective. The bead on the left luminesces much more strongly than the two beads on the right. The white spots on the bead on the left are pixel artifacts.

### EXAMPLE VIII

#### Conjugation of an Antibody with the Europium Macrocycle Labeled PEGA-Bound Peptide of EXAMPLE VII

In this Example, an antibody is coupled to a PEGA-bound peptide. This procedure is based on G. T. Hermanson 1996 (Ref. 26) Chapter 10. Antibody Modification and Conjugation p. 456. The antibody is first selectively reduced to provide two half-molecules, each containing a cysteine, and then this cysteine replaces by disulfide exchange the NpyS group that was part of the PEGA-bound peptide.

#### A. MATERIALS

(a) The europium-macrocycle-labeled-peptide bound to PEGA beads of EXAMPLE VII (EuMac-Peptide-PEGA Beads).

(b) Unconjugated PRB-1 from Phoenix Flow Systems (Anti5BrdU).

(c) EDTA, disodium salt dihydrate, molecular biology grade, Sigma, Catalog No. E5134 (2000-2001).

(d) An aqueous solution containing  $\text{NaH}_2\text{PO}_4$  (0.1 M),  $\text{NaCl}$  (0.15 M), and EDTA (10



1 mM) is titrated with an aqueous solution containing  $\text{Na}_2\text{HPO}_4$  (0.1 M), NaCl (0.15 M), and EDTA (10 mM) to achieve pH 6.0 (Phosphate-EDTA-pH 6.0 buffer).

3 (e) An aqueous solution containing HMTA (0.267 M) and NaCl is (0.15 M), adjusted to pH 7.2 with HCl. Dissolved oxygen is removed from the solution by bubbling nitrogen gas through it (anaerobic chromatography pH 7.2 buffer). The use of this buffer avoids exposure of the lanthanide(III)-macrocycle to either EDTA or phosphate.

8 (f) 2-mercaptoethylamine·HCl, Pierce, Catalog No. 20408 (2000).

10 (g) Tris-Ca Buffer of EXAMPLE IV.

## 11 B. PROCEDURE

13 (a) A chromatography column of Sephadex G-25 is equilibrated with anaerobic chromatography pH 7.2 buffer at 4°C.

15 (b) Ten mg of the Anti5BrdU is added to 1 mL of Phosphate-EDTA-pH 6.0 buffer. 2-mercaptoethylamine·HCl (6 mg) is then added to the Anti5BrdU-containing solution and the mixture is vortex-mixed and incubated for 90 min at 37°C.

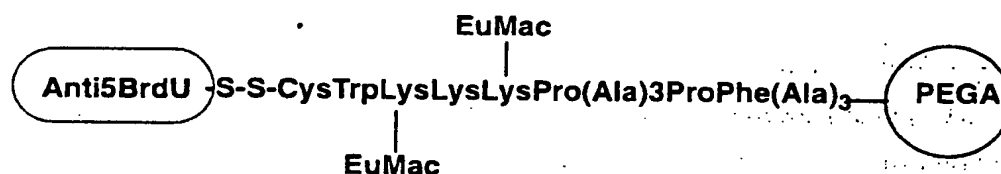
19 (c) The solution containing the reduced Anti5BrdU half molecules with free sulfhydryl groups is added under anaerobic conditions to a Sephadex G-25 column (volume ratio of 1 to 20). This size exclusion chromatography separates the Anti5BrdU from the other components of the reducing solution without reoxidation of the cysteine. The effluent of the column is monitored at 280 nm and the first fractions, which contain the antibody, are pooled.

25 (d) EuMac-Peptide-PEGA Beads are added to a test tube containing a magnetic stirrer. An aliquot of the pooled fractions from (c) containing reduced antibody halves in quantity to provide 5 sulfhydryls per NpyS of conjugated peptide, is added to the EuMac-Peptide-PEGA Beads. The mixture is allowed to react for 20 hours at 4°C with stirring under nitrogen, to form the Anti5BrdU-EuMac-Peptide-PEGA conjugate. The reduced antibody half liberates the S-Npys protecting group from the EuMac-Peptide-PEGA and forms a cystine disulfide bridge. The Peptide-PEGA-Beads are now linked to the antibody by the intervening peptide, Formula XIII. The free 3-nitro-Pyridine-2-thione is incapable of participating in further mixed

1 disulfide formation (Hermanson Chapter 2, 1996 (Ref. 26) p. 151.

2 (e) The suspension of the Anti5BrdU-EuMac-Peptide-PEGA conjugate beads is centri-  
3 fugal at 200 x g for five minutes at 4°C and the supernatant is removed. The removal of the  
4 supernatant minimizes contaminants, such as unbound antibody and free 3-nitro-Pyridine-2-  
5 thione.  
6

7 (f) Tris-Ca Buffer is added to the centrifuge tube of step (e) in the ratio of 0.8 mL per 1  
8 mg of beads and the beads are suspended by tapping. The contents are again centrifuged at  
9 200 x g for five minutes and the supernatant is removed. The wash with Tris-Ca Buffer is  
10 repeated; another aliquot of Tris-Ca Buffer containing 20% glycerol is added and the beads  
11 are stored at -20°C. The structure of the conjugate of AntiBrdU with the EuMac-Peptide-  
12 PEGA is shown by the schematic Formula XIII:



18 Formula XIII

19 The structure of Formula XIII shall be referred to as Anti5BrdU-EuMac-Peptide-PEGA  
20 Conjugate beads  
21

## 22 EXAMPLE IX

### 23 Enzymatic Cleavage of the Antibody Conjugate of the Europium 24 Macrocycle Peptide of EXAMPLE VIII from the PEGA Beads 25

#### 26 A. MATERIALS

27 (a) The Anti5BrdU-EuMac-Peptide-PEGA Conjugate beads of EXAMPLE VIII, previ-  
28 ously washed and suspended and washed in Tris-Ca Buffer.  
29

30 (b) HMTA buffer (0.267 M) adjusted to pH 7.5 with HCl (0.267 M HMTA pH 7.5  
31 Buffer).  
32

1 (c) Microcon YM-10 Centrifugal ultrafiltration unit with an ultrafilter fabricated from  
2 regenerated cellulose with a molecular weight cut-off of 10,000 daltons, Millipore, Catalog  
3 No. 42407, (10,000 mw cut-off filter).

4 B. PROCEDURE  
5

6 (a) The procedures of EXAMPLE IV and EXAMPLE VII are followed except that all  
7 amounts are scaled for the available amount of Anti5BrdU-EuMac-Peptide-PEGA Conjugate  
8 beads. The cleaved EuMac-labeled peptide, with the attached antibody, is removed from the  
9 Peptide-PEGA-Beads by washing with 0.267 M HMTA pH 7.5 buffer.

10 The EuMac-labeled peptide, with the attached antibody, is concentrated by centrifugal fil-  
11 tration with 10,000 mw cut-off filter unit according to the manufacturers literature; it is then  
12 passed through a 0.22 micron pore size membrane filter (Millipore Catalog No. GSWP04700,  
13 2000); 20% glycerol is added, and the solution is stored at -20 °C until use.

14  
15 The combination of EXAMPLE VIII and EXAMPLE IX describe the manufacture of a  
16 product suitable for commercial use, a tagged-analyte-binding species, in this case a labeled  
17 antibody.

18  
19 EXAMPLE X

20 Luminescence Study of a Eu-Macrocycle-Antibody Conjugate Attached  
21 to Apoptotic Cells, Using Gd(III) as Energy Transfer Donor in  
22 Cofluorescence Matrix

23 A. MATERIALS  
24

25 (a) Phoenix Flow Systems APO-BRDU™ Kit, part number AU1001.

26  
27 (b) The EuMac-labeled peptide, with the attached antibody of EXAMPLE IX (EuMac-  
28 Peptide-Anti5BrdU).

29 (c) HMTA 10% aqueous solution adjusted to pH 7.6 with hydrochloric acid (HMTA pH  
30 7.6 buffer).

31  
32 (d) DAPI, Molecular Probes, Catalog No. D-1306 (1999).

1 B. PROCEDURE

2 1. The first part of this procedure consists of the suspension staining of BrdU-contain-  
3 ing cells with EuMac-Antibody and DAPI.

4  
5 (a) The positive and negative control cells of the APO-BRDU™ Kit are resuspended by  
6 swirling the vials. A one mL aliquot of each control cell suspension (approximately  $1 \times 10^6$   
7 cells) is removed and placed in a 12 x 75 mm flow cytometry centrifuge tube. The tubes are  
8 centrifuged (300 x g) for 5 minutes and the 70% (v/v) ethanol supernatant is removed by aspi-  
9 ration, being careful to not disturb the cell pellets.

10  
11 (b) The positive and negative control cells are resuspended in 1 mL of HMTA pH 7.6  
12 buffer containing  $1 \times 10^{-4}$  GdCl<sub>3</sub>. The cells are centrifuged as before and the supernatant is  
13 removed by aspiration.

14 (c) The procedures of step (b) are repeated.

15  
16 (d) The antibody labeling solution is prepared by combining 5 uL of EuMac-Peptide-  
17 Anti5BrdU with 95 uL of the HMTA pH 7.6 buffer.

18  
19 (e) The positive control cell pellets are resuspended in 0.1 mL of the antibody labeling  
20 solution, the centrifuge tube is wrapped with aluminum foil, and the cells are incubated in the  
21 dark for 30 minutes at room temperature.

22 (f) The negative control cell pellets are resuspended in 0.1 mL of the HMTA pH 7.6  
23 buffer, the centrifuge tube is wrapped with aluminum foil, and the cells are incubated in the  
24 dark for 30 minutes at room temperature.

25  
26 (g) 0.9 mL of a 2 uM DAPI solution (0.9 mL of 2 uM solution) is added to the tubes  
27 which contain the positive and negative control cells. The cells are incubated in the dark for a  
28 further 30 minutes at room temperature.

29 2. The second part of this procedure consists of the centrifugal cytology and fluores-  
30 cence microscopy of the dual stained cells.

31 (a) A 1 mL sample of each of the two cell suspensions of step (1.g) is decanted into a  
32

1 Leif Centrifugal Cytology Bucket, R. C. Leif, 2000 (Ref. 44) and is centrifuged at 300 x g for  
2 ten minutes at room temperature. The cells are sedimented onto and bound to an aminosilane  
3 treated slide, Labscientific, Inc. Livingston, N.Y.

4 (b) The supernatants are removed by aspiration from the Centrifugal Cytology Bucket  
5 sample block; and 0.2 mL of the cofluorescence solution is added to the fixative chambers  
6 connecting to the cell containing sample chambers of the Centrifugal Cytology Bucket sample  
7 block.  
8

9 (c) The Centrifugal Cytology Bucket is centrifuged at 300 x g for five minutes at room  
10 temperature, the sample block is separated from the slide, and a cover-glass is placed over the  
11 dispersions of fixed, stained cells.

12 (d) The cells are then viewed with a fluorescence microscope under episcopic illumina-  
13 tion with mercury arc excitation. The excitation filter passes 365 nm light, which is reflected  
14 by a 400 nm dichroic mirror and excites the europium macrocycle. The emitted red light  
15 passes through the dichroic mirror and a 619 nm narrow band-pass filter. The EuMac-Peptide-  
16 Anti5BrdU bound to the incorporated 5BrdU is then observed and measured. The DAPI  
17 stained DNA in the nucleus is observed or measured through a broad-band emission 450 nm  
18 filter. The positive control cells show both a strong red and a blue nuclear emission; the nega-  
19 tive control cells show only a blue nuclear emission. Surprisingly, no background binding of  
20 the EuMac-Peptide-Anti5BrdU is detected. The  $1.20 \times 10^{-4}$  M. Gd(III) cation of the cofluores-  
21 cence solution blocks the nonspecific binding of the positively charged EuMac.  
22

23 EXAMPLE X describes a cytological assay based on a commercially available kit with the  
24 use of a tagged-analyte-binding species, in this case a labeled antibody.  
25

#### 26 EXAMPLE XI

##### 27 Simultaneous use of Two Lanthanide Tags as Secondary Reagents for 28 Comparative Genomic Hybridization Measurements 29

30 In this Example, methods of this invention to analyze genomes by Comparative Genomic  
31 Hybridization (CGH) are exemplified by employing two luminescence species that are each  
32

1 attached to a secondary reagent. This procedure is based on US Patent 5,976,790. Pinkel et al.  
2 (Ref. 45) which describes the following steps for CGH:

- 3 1. Removal of Repetitive Sequences and/or Disabling the Hybridization Capacity of  
4 Repetitive Sequences.
- 5 2. Labeling the Nucleic Acid Fragments of the Subject Nucleic Acids.
- 6 3. In Situ Hybridization.

8 Pinkel et al. 1999 (Ref. 45) summarize In Situ Hybridization as: "Generally in situ hybrid-  
9 ization comprises the following major steps: (1) fixation of tissue or biological structure to be  
10 examined, (2) prehybridization treatment of the biological structure to increase accessibility  
11 of target DNA, and to reduce nonspecific binding, (3) hybridization of the mixture of nucleic  
12 acids to the nucleic acid in the biological structure or tissue; (4) posthybridization washes to  
13 remove nucleic acid fragments not bound in the hybridization and (5) detection of the hybrid-  
14 ized nucleic acid fragments."

15  
16 These authors state that their present technique is limited: "At the current stage of develop-  
17 ment of CGH, sensitivity is primarily limited by the granularity of the hybridization signals in  
18 the metaphase chromosomes. Further improvements in sensitivity will be achieved by optimi-  
19 zation of the probe concentration and labeling, and by the averaging of the green-to-red fluo-  
20 rescence ratios from several metaphase spreads."

## 21 A. MATERIALS

22  
23 (a) SmMac-mono-NCS is synthesized according to the procedures of Examples XI and  
24 XXXVI B Step 1 of patent 5,696,240, with the substitution of Sm(III) for Eu(III).

25  
26 (b) SmMac-labeled peptide with attached avidin is produced by the procedures of  
27 EXAMPLE VIII and EXAMPLE IX, with the substitution of the SmMac-mono-NCS for the  
28 EuMac-mono-NCS and the substitution of Avidin for Anti5BrdU. The SmMac-labeled pep-  
29 tide with attached Avidin will be referred to as SmMac-Peptide-Avidin.

30 (c) The EuMac-labeled peptide with attached anti-digoxigenin, is produced by the proce-  
31 dures of EXAMPLE VIII and EXAMPLE IX with the substitution of anti-digoxigenin for  
32 Anti5BrdU. This peptide will be referred to as EuMac-Peptide-anti-digoxigenin.

1 (d) All other materials are as described in US Patent 5,976,790

2 B. PROCEDURE

3  
4 (a) The procedure of Kallioniemi et al. 1994 (Ref. 46) is followed. The target metaphase  
5 slides are prepared from phytohemagglutinin-stimulated peripheral blood lymphocytes from a  
6 normal male. To assess the hybridization characteristics, each batch of slides is extensively  
7 tested with labeled normal genomic DNA and with whole chromosome-painting probes. If  
8 evidence of dim or non-uniform hybridization is detected, the entire batch of slides is aban-  
9 doned, and another batch is prepared.

10 (b) A DNA sample from abnormal tissue is labeled with biotin-14dATP (test sample). A  
11 second DNA sample from normal tissue is labeled with digoxigenin-11-dUTP (normal refer-  
12 ence DNA) using the Bionick labeling system (BRL).  
13

14 (c) The amounts of DNase and DNA polymerase I are adjusted so that the probe-frag-  
15 ment-size distribution after labeling is 600-2000 base pairs (a smear in a nondenaturing agar-  
16 ose gel). Probe fragments of this size are necessary to obtain uniform, intense hybridization.  
17

18 (d) Sixty to 100 ng of each of the labeled probes and 5 ug of unlabeled Cot-I DNA are  
19 precipitated with ethanol.

20 (e) The DNAs are dissolved in 10 uL of hybridization buffer [50% (vol/vol) formamide/  
21 10% (wt/vol) dextran sulfate/2x standard saline/citrate, pH 7], denatured at 70°C for 5 min,  
22 and incubated at 37°C for 30 min.  
23

24 (f) Metaphase slides are denatured in 70% formamide/2 x standard saline/citrate, pH 7 at  
25 70°C for 3 min, dehydrated sequentially in 70%, 85%, and 100% ethanol, treated with Pro-  
26 teinase K (0.1 ug/mL in 20 mM Tris/2 mM CaCl<sub>2</sub>, pH 7.5) at 37°C for 7.5 min, and dehy-  
27 drated again.  
28

29 (g) The hybridization mixture is applied on slides and hybridized for 2-3 days at 37°C in  
30 a moist chamber.

31 (h) After hybridization, the slides are washed and stained by using a single layer of  
32

1 SmMac-Peptide-Avidin (to visualize bound biotinylated probes) at 5 ug/mL and EuMac-Pep-  
2 tide-anti-digoxigenin at 1 ug/mL (to visualize bound digoxigenin-labeled probes).

3 (i) Samples are counterstained with DAPI in an anti-fade solution.

4  
5 (j) The slide is dipped in the cofluorescence solution and a coverslip is applied.

6  
7 (k) The chromosomes are imaged and their emission intensity is measured with a fluores-  
8 cence microscope with episcopic illumination and equipped with a digitized camera. The 365  
9 nm exciting radiation from a mercury lamp is separated from the luminescence emission of  
10 the chromosomes by a dichroic mirror that reflects half the light at 400nm. The movable emis-  
11 sion filter holder has at least 3 filters: a wide band 450 filter for DAPI, a narrow 619 nm filter  
12 for the Eu(III) emission, and a 599 and 644 nm filter for the Sm(III) emission. The band  
13 widths of the emission filters are 10 nm full-width at half maximum.

14 (l) The individual chromosomes are identified by the DAPI banding and their size. The  
15 signal-to-noise ratio of both the Eu(III) and Sm(III) emission, and the lack of overlap between  
16 the two spectra, increases the precision of the measurements permitting probe-fragments  
17 smaller than 600 base pairs to be used and eliminating the need for signal averaging from mul-  
18 tiple chromosomes of the same type.

## 19 EXAMPLE XII

### 20 Synthesis of A Europium Macrocycle Labeled Peptide-Substituted 21 Polynucleotide

#### 22 A. MATERIALS

23  
24 (a) The Proteinase K cleavable peptide shown in Formula XIV is synthesized employing  
25 an amino-PEGA support similar to that described EXAMPLE IV, Peptide-PEGA-Beads. A  
26 schematic representation of the second lot of the Peptide-PEGA-Beads is shown in Formula  
27 XIV:  
28





Formula XIV

6 The peptide in Formula XIV has the number of lysines increased from the 3 of EXAMPLE  
7 IV to 5 and a spacer amino acid, alanine, interspersed to facilitate both the reaction of the  
8 EuMac-mono-NCS with the lysines and the subsequent interaction with the cofluorescence  
9 solution.

10  
11 B. PROCEDURE

12 An oligonucleotide carrying a EuMac-labeled-polypeptide tail is synthesized by the proce-  
13 dure developed by Haralambidis et al. 1990A (Ref. 4) for the synthesis of carboxyfluorescein  
14 conjugates of both peptide-oligodeoxyribo-nucleotides and polyamide-oligonucleotides.  
15 According to this procedure, which employs a commercially available automated DNA syn-  
16 thesizer (Applied Biosystems Inc.), the following steps are performed:

17  
18 (a) The terminal amino group of the Peptide-PEGA-Beads is converted to an amide by  
19 reaction with an  $\alpha,\omega$ -hydroxycarboxylic acid derivative, the structure 2 of Haralambidis et al.  
20 1990A. The hydroxyl group of the acid is previously protected by conversion to a 9-phenylx-  
21 anthene-9-yl (pixyl) ether and the carboxyl terminus is activated as the p-nitrophenyl ester.

22 (b) The hydroxyl group which now terminates the peptide is deprotected; it is then ester-  
23 ified with a phosphoramidate, and the bead-linked-peptide-conjugated polynucleotide is sub-  
24 sequently assembled by sequential reaction with methyl N,N-diisopropyl nucleoside  
25 phosphoramidates to a 30mer. This 30mer oligonucleotide is described by Haralambidis et al.  
26 1990A as being d(GGGCTTCACAACATCTGTGATGTCAGCAGG). Protected lysine resi-  
27 dues are included in both the peptide and the polyamide to provide primary amino functional-  
28 ities suitable for conjugation with an isothiocyanate.

29  
30 (c) The primary amino groups of the lysine residues of the bead-linked-peptide-conju-  
31 gated polynucleotide are deprotected and the lysines are coupled to multiple EuMac-mono-  
32 NCS according to the procedures of EXAMPLE VII.

1 (d) The EuMac-labeled-bead-linked-peptide-conjugated polynucleotide is released from  
2 the PEGA beads by enzymatic hydrolysis with Proteinase K by the procedures of EXAMPLE  
3 VII

### 4 EXAMPLE XIII

#### 5 Hybridization and Detection of a Europium Macrocyclic Labeled 6 Peptide-Substituted Polynucleotide

##### 7 A. MATERIALS

8  
9  
10 (a) The EuMac-labeled-bead-linked-peptide-conjugated polynucleotide of EXAMPLE  
11 XII (EuMac-Peptide-Polynucleotide).

12 (b) An aqueous solution containing NaCl (0.75 M), M sodium citrate (0.075M),  
13  $\text{NaH}_2\text{PO}_4$  (25 mM),  $\text{Na}_2\text{HPO}_4$  (25 mM), tetrasodium pyrophosphate (10 mM), disodium ade-  
14 nosine triphosphate (0.1 mM) Sigma, Catalog No. A 7699 (1998), salmon testes DNA (25 mg/  
15 L, Sigma, Catalog No. D 1626 (1998), Ficoll (0.01% w/v), Sigma, Catalog No. F 2637 (1998),  
16 polyvinylpyrrolidone (0.01%), Sigma, Catalog No. PD 5288 (1998), bovine serum albumin  
17 (0.01%), Sigma, Catalog No. B 4287 (1998), and 20% N,N-dimethylformamide, Sigma, Cata-  
18 log No. D 7656 (1998), (hybridization buffer).  
19

20 (c) The a 3.7 Kb plasmid derived from pUC and containing a 1 kb mouse renal kallikrein  
21 cDNA insert of Haralambidis et al. 1990B (Ref. 5) (Plasmid Positive Control).

22 (d) The similar pUC plasmid containing the metallothionein IIA gene promoter spliced  
23 with the chloramphenicol acetyl transferase (CAT) structural gene of Haralambidis et al.  
24 1990B (Ref. 5) (Plasmid Negative Control).  
25

26 (e) Herring sperm DNA, Sigma, Catalog No. D 7290 (1998).  
27

28 (f) Nitrocellulose membranes (Sigma, Catalog No. Z36,022-8 (1998)).  
29

##### 30 B. PROCEDURE

31 The procedures of Haralambidis et al. 1990B (Ref. 5) are followed with the exception of  
32 the substitution of the EuMac-Peptide-Polynucleotide for the fluorescein-labeled peptide-sub-

stituted polynucleotide of Haralambidis et al. 1990B (Ref. 5). Hybridization experiments with the EuMac-Peptide-Polynucleotide conjugate probes are carried out onto dot blots containing 3.7 kb plasmid positive and negative controls. Each dot contains also 1 ug of herring sperm DNA.

(a) The nitrocellulose membranes are prehybridized at 42°C for 6.5 h in 10 mL of hybridization buffer.

(b) 100 ng of the EuMac-Peptide-Polynucleotide is then added and it is allowed to hybridize at 42°C overnight.

(c) The filters are washed four times, for ten minutes each, at 42°C in 0.2xSSC (0.03 M NaCl, 0.003 M sodium citrate).

(d) The filters are gently wetted with the cofluorescence solution and allowed to air-dry.

(e) The filters are examined under ultraviolet light (365 nm band). The Plasmid Positive Control emits a red glow. The emission of the Plasmid Negative Control is much weaker.

These results with the lanthanide(III) complexes show an additive effect; the luminescence is proportional to the total number of lanthanide(III) macrocycles bound to the peptide. This is totally different from the extremely low (0.05 to 0.002) ratio between the fluorescence of fluorescein tags on a polymer and the fluorescence of the fluorescein monomer observed by Haralambidis et al. 1990B (Ref. 5). Thus, where a conventional organic fluorophore did not work, an example of the new tagged-analyte-binding species will work; specifically multiple lanthanide(III) macrocycles (EuMac) bound to a peptide.

The Peptide-PEGA-Beads with free hydroxyl groups which are formed by converting the alpha amino groups into an amide by reaction with an  $\alpha,\omega$ -hydroxycarboxylic acid derivative can be stored. If tags that are stable to the nucleic acid synthesis and deprotection reactions are used, tagged Peptide-PEGA-Beads with free hydroxyl groups can be stored and subsequently extended. The use of an enzymatic cleavage minimizes the degradation of tags that can not withstand harsh treatments. The free hydroxyl groups can be extended with short nucleotide sequences, which after binding to a complementary region of a large template can be enzymatically extended (Strachan and A. P. Read, 1999)

References:

- 1
- 2 1. A. Schwartz and E. Fernandez-Repollet, "Development of clinical standards for flow  
3 cytometry", *Annals N.Y. Academy of Science* 677 pp. 28-39, 1993.
- 4 2. H.M. Shapiro, *Practical Flow Cytometry*, Third Edition p. 91, 277 Wiley-Liss, New York,  
5 N. Y 1995.
- 6 3. J. R. Lakowicz, "Principles of Fluorescence Spectroscopy", Plenum Press, 1983.
- 7 4. J. Haralambidis, L. Duncan, K. Angus, and G. W. Tregear, "The Synthesis of Polyamide-  
8 Oligonucleotide Conjugate Molecules", *Nucleic Acids Research* 18, pp. 493-499, 1990 A.
- 9 5. J. Haralambidis, K. Angus, S. Pownall, L. Duncan, M. Chai, and G. W. Tregear, "The Prep-  
10 aration of Polyamide-Oligonucleotide Probes Containing Multiple Non-radioactive Labels",  
11 *Nucleic Acids Research* 18, pp. 501-505, 1990 B.
- 12 6. *Handbook of Fluorescent Probes and Research Chemicals*, 6th edition, R. P. Haugland,  
13 Molecular Probes, Inc., Eugene, Oreg. 97402, 1996.
- 14 7. Sigma, *Biochemicals and Reagents for Life Science Research*, Sigma-Aldrich 2000-2001.
- 15 8. O. Siiman, C. Smith, P. Roth, A. Bm-shteyn, R. Raynor, US Patent 5,891,741, "Antibody-  
16 aminodextran-phycoobiliprotein Conjugates", 1999.
- 17 9. Peterson J. J. and Meares C. F., "Cathepsin Substrates as Cleavable Peptide Linkers in Bio-  
18 conjugates, Selected from a Fluorescence Quench Combinatorial Library", *Bioconjugate*  
19 *Chemistry* 9, pp. 618-626, 1998.
- 20 10. Peterson J. J. and Meares C. F., "Enzymatic Cleavage of Peptide-Linked Radiolabels from  
21 Immunoconjugates", *Bioconjugate Chemistry* 10, pp. 553-557, 1999.
- 22 11. H. Takalo, V.-M Mikkala, H. Mikola, P. Litti, and I. Hemmila, Synthesis of europium(III)  
23 chelates suitable for labeling of bioactive molecules", *Bioconjugate Chemistry* 5, pp. 278-  
24 282, 1994.
- 25 12. Lamture JB, Wensel TG., "Intensely luminescent immunoreactive conjugates of proteins  
26 and dipicolinate-based polymeric Tb (III) chelates", *Bioconjug Chem.* 6, pp. 88-92, 1995.
- 27 13. Kwiatkowski M, Samiotaki M, Lamminmaki U, Mikkala VM, Landegren U., "Solid-  
28 phase synthesis of chelate-labeled oligonucleotides: application in triple-color ligase-medi-  
29 ated gene analysis", *Nucleic Acids Research* 22, pp. 2604-2611, 1994.
- 30 14. H. Salo, A. Guzaev, H. Lonnberg, "Disulfide-Tethered Solid Supports for Synthesis of  
31 Photoluminescent Oligonucleotide Conjugates: Hydrolytic Stability and Labeling on the Sup-  
32 port", *Bioconjugate Chemistry* 9, pp 365-371, 1998.
15. D. A. Zarling, M. J. Rossi, N. A. Peppers, J. Kane, G. W. Faris, M. J. Dyer, S. Y. Ng, and

- 1 L. V. Schneider, US Patent 5,736,410, "Up-Converting Reporters for Biological and Other  
2 Assays Using Laser Excitation Techniques", 1998.
- 3 16. L. M. Vallarino and R. C. Leif, U.S. Patent 5,373,093, "Macrocycle complexes of  
4 Yttrium, the Lanthanides and the Actinides having Peripheral Coupling Functionalities",  
5 1994.
- 6 17. L. M. Vallarino and R. C. Leif, US Patent 5,696,240, "Macrocycle complexes of Yttrium,  
7 the Lanthanides and the Actinides having Peripheral Coupling Functionalities Continuation-  
8 In-Part", 1997.
- 9 18. R. C. Leif, P. M. Harlow, and L. M. Vallarino, "Production, Fixation, and Staining of  
10 Cells on Slides for Maximum Photometric Sensitivity". Proceedings of Biochemical Diagnos-  
11 tic Instrumentation, Progress in Biomedical Optics. R. F. Bonner, G. E. Cohn, T. M. Laue, and  
12 A. V. Priezzhev Eds.; SPIE Proceedings Series 2136, pp. 255-262, 1994.
- 13 19. N. Sabbatini, L. De Cola, L.M. Vallarino, and G. Blasse, "Radiative and Nonradiative  
14 Transitions in the Eu(III) Hexaaza Macrocyclic Complex  
15 [Eu(C<sub>22</sub>H<sub>26</sub>N<sub>6</sub>)(CH<sub>3</sub>COO)](CH<sub>3</sub>COO)Cl 2H<sub>2</sub>O," *J. Phys. Chem.*, Vol. 91, pp. 4681-4685,  
16 1987.
- 17 20. A. J. Bromm Jr., R. C. Leif, J. R. Quagliano, and L. M. Vallarino, "The Addition of a Sec-  
18 ond Lanthanide Ion to Increase the Luminescence of Europium(III) Macrocyclic Complexes",  
19 Proceedings of Optical Diagnostics of Living Cells II, D. L. Farkas, R. C. Leif, B. J. Trom-  
20 berg, Editors, SPIE Progress in Biomedical Optics, A. Katzir series Editor, Vol. 3604, ISBN  
21 0-8194-3074-9, pp. 263-272, 1999.
- 22 21. J. R. Quagliano, R. C. Leif, L. M. Vallarino, and Steven A. Williams, "Methods to  
23 Increase the Luminescence of Lanthanide(III) Macrocyclic Complexes", Optical Diagnostics  
24 of Living Cells III, D. L. Farkas and R. C. Leif, Editors, Proceedings of SPIE Vol. 3921. pp.  
25 124-133, 2000.
- 26 22. A. M. Adeyiga, P. M. Harlow, L. M. Vallarino, and R. C. Leif, "Advances in the Develop-  
27 ment of Lanthanide Macrocyclic Complexes as Luminescent Bio-Markers". Advanced Tech-  
28 niques in Analytical Cytology, Optical Diagnosis of Living Cells and Biofluids, T. Askura, D.  
29 L. Farkas, R. C. Leif, A. V. Priezzhev, and B. J. Tromberg Eds.; A. Katzir Series Editor,  
30 Progress Biomedical Optics Series Editor SPIE Proceedings Series, Vol. 2678, pp. 212-220,  
31 1996.
- 32 23. R. C. Leif and L. M. Vallarino, "Rare-Earth Chelates as Fluorescent Markers in Cell Sep-  
aration and Analysis". ACS Symposium Series 464, Cell Separation Science and Technology,  
D. S. Kompala and P. W. Todd Editors, American Chemical Society, Washington, DC, pp 41-  
58, 1991.
24. R. B. Merrifield, *J. Am. Chem. Soc.*, 85, pp. 2149, 1963.
25. P. Lloyd-Williams, F Albericio, and E. Giralt, "Chemical Approaches to the Synthesis of

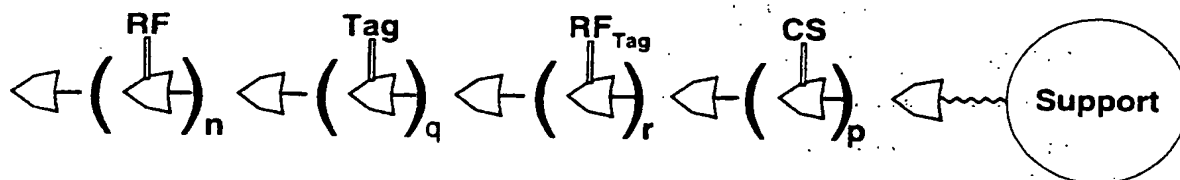
- 1 Peptides and Proteins," CRC Press, ISBN 0-8493-9142-3, 1997.
- 2 26. G. T. Hermanson, Bioconjugate Techniques," Academic Press, ISBN0-12-342336-8,  
3 1996.
- 4 27. C. C. Barrett and D. T. Elmore, "Amino Acids and Peptides, Cambridge University Press,  
5 ISBN 0 521 46827 2, 1998.
- 6 28. L. Pauling, The Nature of the Chemical Bond, Third Edition, Cornell University Press,  
7 1960.
- 8 29. T. Strachan and A. P. Read, "Human Molecular Genetics 2nd ed", Wiley-Liss, ISBN 0-  
9 471-33061-2, 1999.
- 10 30. J. R. Kettman, T. Davies, D. Chandler, K.G. Oliver, R.J. Fulton, "Classification and prop-  
11 erties of 64 multiplexed microsphere sets", Cytometry 33, pp. 234-243, 1998.
- 12 31. L. Stryer and R. P. Haugland, "Energy transfer: a spectroscopic ruler." Proc Natl Acad Sci  
13 USA 58, pp 719, 1967.
- 14 32 Y. Li and A. n. Glazer, "Design, synthesis, and Spectroscopic Properties of Peptide-  
15 Bridged Fluorescence Energy-Transfer Cassettes," Bioconjugate Chemistry 10, pp241-245  
16 (1999)
- 17 33. Waggoner, A.S., L.A. Ernst, C.-H. Chen, and D.J. Rechtenwald. "PE-CY5: A new fluo-  
18 rescent antibody label for three-color flow cytometry with a single laser." Ann. N.Y. Acad.  
19 Sci. 677: 185 - 193, 1993.
- 20 34. <http://www.coulter.com/Coulter/Cytometry/Catalog/SS000152.asp>
- 21 35. Rechtenwald, D.J. United States Patent No. 4,876,910, "Peridinin-chlorophyll complex as  
22 fluorescent label", 1989.
- 23 36. E. Gurr, "Synthetic Dyes in Biology, Medicine and Chemistry", Academic Press, SBN:  
24 12-309650-2, 1971.
- 25 37. J. March, "Advanced Organic Chemistry Reactions, Mechanisms and Structure", 3rd Ed.,  
26 J. Wiley & Sons, New York, 1985.
- 27 38. R. C. Leif, R. A. Thomas, T. A. Yopp, B. D. Watson, V. R. Guarino D. H. K. Hindman, N.  
28 Lefkove and L. M. Vallarino, "Development of Instrumentation and Fluorochromes for Auto-  
29 mated Multiparameter Analysis of Cells". Clin. Chem. 23, pp. 1492-1498, 1977.
- 30 39. R. C. Leif, S. P. Clay, H. G. Gratzner, H. G. Haines, K. V. Rao and L. M. Vallarino,  
31 "Markers for Instrumental Evaluation of Cells of the Female Reproductive Tract: Existing and  
32 New Markers". The Automation of Uterine Cancer Cytology, G. L. Wied, G. F. Bahr and P.  
H. Bartels Eds.; Tutorials of Cytology, Chicago, pp. 313-344, 1976.

- 1 40. W. H. Wright, G. A. Rundle, N. A. Mufti, Y.-M. M. Yao, D. E. Cooper, "Flow Cytometry  
2 with Upconverting Phosphor Reporters". Optical Investigations of Cells In Vitro and In Vivo,  
3 D. L. Farkas, R. C. Leif, and B. J. Tromberg Eds.; A. Katzir Series Editor, Progress Biomed-  
4 ical Optics. Vol. 3260, pp. 245-254, 1998.
- 5 41. J. March, "Advanced Organic Chemistry Reactions, Mechanisms and Structure", 3rd Ed.,  
6 J. Wiley & Sons, New York, pp. 361-363, 1985.
- 7 42. K. Bromme, K. Peters, S. Fink, and S. Fittau, "Enzyme-Substrate Interactions in the  
8 Hydrolysis of Peptide Substrates by Thermolysin, Subtilisin BPN, and Proteinase K", Archives  
9 of Biochemistry and Biophysics, 244, pp. 439-446, 1986.
- 10 43. G. Mezo, N. Mihala, D. Andreu, and F. Hudecz, "Conjugation of Epitope Peptides with  
11 SH Group to Branched Chain Polymeric Polypeptides via Cys(Npys)", Bioconjugate Chemis-  
12 try, 11, pp. 484-491, 2000
- 13 44. R. C. Leif "Methods for Preparing Sorted Cells as Monolayer Specimens". In Living :  
14 Color, Protocols in Flow Cytometry and Cell Sorting, Eds. R. A. Diamond and S. DeMaggio,  
15 Springer, ISBN 3-540-65149-7, pp. 592-619, 2000.
- 16 45. D. Pinkel, J. W. Gray, A. Kallioniemi, O-P Kallioniemi, F. Waldman, and M. Sakamoto,  
17 US Patent 5,976,790, "Comparative Genomic Hybridization (CGH)", 1999.
- 18 46. A. Kallioniemi, O-P. Kallioniemi, J. Piper, M. Tanner, T. Stokke, L. Chen, H. S. Smith, D.  
19 Pinkel, J. W. Gray, And F. M. Waldman, "Detection and Mapping of Amplified DNA  
20 Sequences in Breast Cancer by Comparative Genomic Hybridization"; Proc. Natl. Acad. Sci.  
21 USA. 91, pp. 2156-2160, 1994.
- 22
- 23
- 24
- 25
- 26
- 27
- 28
- 29
- 30
- 31
- 32

## CLAIMS

1. A water-soluble polymer linked to a solid support and selectively cleavable therefrom, comprising closest to said support a cleavage segment, of known composition and sequence made up of at least one monomer unit; a second segment of known composition and sequence separated from said support by at least said cleavage section and including at least one or more monomer units selected from the group consisting of monomer units linked to a reactive functionality able to be covalently coupled to a tag and monomer units linked to a tag, and a third segment of known composition and sequence separated from said support by at least said cleavage section and including at least one monomer unit linked to a reactive functionality capable of forming a covalent bond with an analyte-binding species or an analyte.

2. The polymer of Claim 1 represented by the schematic Formula IV:



Formula IV

wherein each left pointing broad-arrow shape represents a monomer unit; RF independently represents a reactive functionality linked to a monomer unit and serving to bind to an analyte-binding species; RF<sub>tag</sub> independently at each occurrence represents a reactive functionality able to be covalently coupled to a tag; Tag independently at each occurrence represents an optical-label, or other-label, or separation-tag linked to a monomer unit; CS represents at least one monomer unit constituting the cleavable link to the support shown by the circular shape at the right; broad-arrow shapes without other indication represent spacer monomer units, which need not be present; n is a number from 1 to 10; r is a number from 0 to 1,000, q is a number from 0 to 1,000; provided that the sum of r and q is a number from 1 to 1,000; and p is a number from 1 to 25.

3. The polymer of Claim 2 in which q is zero.

4. The polymer of Claim 2 in which r is zero.



5. The polymer of Claim 2 in which q is a number from 1 to 1,000 and r is a number from 1 to 1,000 -q.
- 6 A tagged water-soluble polymer linked to a solid support and selectively cleavable therefrom, comprising a cleavage segment of known composition and sequence consisting of at least one monomer unit; a second segment of known composition and sequence including at least one monomer unit linked to a reactive functionality able to be covalently coupled to a tag, provided at least one such monomer unit is coupled to a tag; and a third segment of known composition and sequence including at least one monomer unit linked to a reactive functionality capable of forming a covalent bond with an analyte-binding species or an analyte.
7. The polymer of Claim 1, wherein said solid support is a water-insoluble, swellable functionalized bead having a dry particle size in the range from 10 to 500 microns.
8. The polymer of Claim 7 wherein said solid support has attached multiple, functionalized hydrophilic polymer side chains.
9. The polymer of Claim 1, wherein said solid support is a cross-linked hydrophilic swellable functionalized bead having a wet particle size in the range from 10 to 500 microns.
10. The polymer of Claim 9, wherein said solid support has attached multiple, functionalized hydrophilic polymer side chains.
11. The polymer of Claim 8, wherein said hydrophilic polymer side chains are polyethylene glycol.
12. The polymer of Claim 11, wherein said support is at least one (2-acrylamidopropyl) substituted poly(ethylene glycol).
13. The polymer of Claim 12, wherein said support is acryloylated bis(2-aminopropyl)polyethylene glycol/dimethyl acrylamide copolymer.
14. The polymer of Claim 1, wherein the functionality at the attachment site of said solid support to said polymer can react with the carboxyl group of an amino acid.
15. The polymer of Claim 1, wherein the functionality at the attachment site of said solid support to said polymer can react with the amino group of an amino acid.

16. The polymer of Claim 1, wherein the functionality at the attachment site of said solid support to said polymer can react with the 5'Phosphate of a nucleotide.
17. The polymer of Claim 1, wherein the functionality at the attachment site of said solid support to said polymer can react with the 3'hydroxyl of a nucleotide.
18. The polymer of Claim 1, wherein the functionality at the attachment site of said solid support to said polymer is selected from the group consisting of amino, azide, alcoholic hydroxyl, phenolic hydroxyl, aldehyde, carboxylic acid, carboxamide, halogen, isocyanate, isothiocyanate, mercapto, nitrile, functionalized alkyl, functionalized aryl, and functionalized alkyl-substituted aryl substituents.
19. The polymer of Claim 18, wherein the functionality on said support is an amino group.
20. The polymer of Claim 18, wherein the functionality on said support is a carboxyl group.
21. The polymer of Claim 18, wherein the functionality on said support is a hydroxyl group.
22. The polymer of Claim 1 comprising amino acid monomer units linked by an amide linkage.
23. The polymer of Claim 22 wherein said amino acid monomers comprise naturally occurring amino acids and synthetic amino acids.
24. The polymer of Claim 22 wherein said amino acid monomers comprise L amino acids and D amino acids.
25. The polymer of Claim 22, wherein at least one aminoacid monomer unit absorbs light in the range from 200 to 300 nm.
26. The polymer of Claim 22, wherein at least one monomer unit is tryptophan.
27. The polymer of Claim 22, wherein at least one amino acid monomer unit has a reactive functionality.
28. The polymer of Claim 27, wherein said reactive functionality is selected from the group consisting of free amino groups, protected amino groups, free carboxyl groups, protected carboxyl groups, free hydroxyl groups, protected hydroxyl groups, free mercapto groups and protected mercapto groups.

29. The polymer of Claim 28, wherein at least one reactive functionality is an amino group.
30. The polymer of Claim 28, wherein a further reactive functionality is a mercaptan group or a disulfide group.
31. The polymer of Claim 30, wherein said reactive functionality is 3-nitro-2-pyridinesulphenyl (NpyS) group bound to the cysteine by a disulfide link.
32. The polymer of Claim 27, wherein said reactive functionality is an alkyl or aryl halide.
33. The polymer of Claim 27, wherein at least one reactive functionality is linked to an analyte-binding species.
34. The polymer of Claim 1 wherein said water-soluble polymer consists essentially of nucleotide monophosphate monomer units.
35. The polymer of Claim 1 wherein said water-soluble polymer consists essentially of sugar monomer units.
36. The polymer of Claim 1 wherein said water-soluble polymer consists essentially of monomer units having a functionalized side chain able to undergo sequential synthesis on a solid support.
37. The polymer of Claim 1, wherein said cleavage segment comprises a sequence of at least two linked monomer units that is selectively cleavable from said support in the presence of an enzyme.
38. The polymer of Claim 1, wherein said cleavage segment comprises one or more disulfide linked monomer units that is selectively cleavable from said support in the presence of a reducing agent.
39. The polymer of Claim 37, wherein said sequence comprises linked amino acid monomer units.
40. The polymer of Claim 37, wherein said enzyme is a proteinase.
41. The polymer of Claim 40, wherein said proteinase is Proteinase K.
42. The polymer of Claim 37, wherein said sequence comprises linked nucleotides.

43. The polymer of Claim 37, wherein said enzyme is an endonuclease.
44. The polymer of Claim 43 wherein said endonuclease is a restriction endonuclease.
45. The polymer of Claim 44 wherein said restriction endonuclease is a rare-cutter.
46. The polymer of Claim of 45 wherein said rare-cutter is BssHII from *Bacillus stearothermophilis*.
47. The polymer of Claim of 45 wherein said rare-cutter is NotI from *Nordcacia otitidis-caviarum*.
48. The polymer of Claim 6, wherein at least one tag is an optical-label, an other-label, or a separation-tag.
49. The polymer of Claim 48, wherein said optical-label is capable of absorbing and/or emitting light in the wavelength range from 200 to 1400 nanometers.
50. The polymer of Claim 49, wherein said optical-label is capable of absorbing light in a certain wavelength range and emitting light in a higher wavelength range.
51. The polymer of Claim 50, wherein said optical-label is capable of emitting light in the range from 300 to 1,400 nanometers.
52. The polymer of Claim 50, wherein said optical-label is capable of absorbing light in the range from 200 to 1,000 nanometers.
53. The polymer of Claim 48, comprising at least two different optical-labeled monomer units.
54. The polymer of Claim 49, wherein at least one optical-label is capable of transferring energy to a second optical-label.
55. The polymer of Claim 53, wherein the sequence of monomer units comprises monomer units to which are attached said optical-labels and at least one spacer monomer unit such that said first optical-label of Claim 53 has a geometry relative to said second optical label to maximize energy transfer between said optical-labels.
56. The polymer of Claim 49, wherein at least one optical-label is a lanthanide compound.

57. The polymer of Claim 56, wherein said lanthanide compound is a lanthanide complex.
58. The polymer of Claim 57, wherein said lanthanide complex is a lanthanide macrocycle.
59. The polymer of Claim 56, wherein said lanthanide compound is a compound of europium, samarium, terbium, or dysprosium.
60. The polymer of Claim 56, wherein said lanthanide compound is an energy transfer acceptor lanthanide macrocycle compound having an emission spectrum maximum in the range from 500 to 950 nanometers.
61. The polymer of Claim 56, wherein said lanthanide compound is an energy transfer acceptor lanthanide macrocycle compound having an excitation spectrum maximum in the range from 200 to 700 nanometers.
62. The polymer of Claim 56 where said lanthanide complex is an energy transfer acceptor lanthanide element macrocycle compound having an emission spectrum peak in the range from 500 to 950 nanometers.
63. The polymer of Claim 56, wherein said lanthanide complex accepts energy from a luminescence enhancer.
64. The polymer of Claim 63, wherein said luminescence enhancer is free in solution.
65. The polymer of Claim 63 wherein a second lanthanide ion is involved with the transfer of energy to said lanthanide complex.
66. The polymer of Claim 53, wherein said first optical-label is a luminescence enhancer and said second optical-label is a lanthanide complex.
67. The polymer of Claim 55, wherein said luminescence enhancer and said second optical-label is a lanthanide complex having geometry relative to said enhancer so as to maximize the emission of light.
68. The polymer of Claim 66, wherein said luminescence enhancer is a beta-diketone or beta-diketonate or a mixture thereof.

69. The polymer of Claim 54 wherein the first optical-label and second optical-label are an organic optical-label pair.

70 The polymer of Claim 69 wherein the second optical-label after receiving energy from the first optical-label, emits light in the range of 300 to 1,400nm.

71. The polymer of Claim 54, wherein the order of the monomer units linked to said optical-labels is such that there are three or more different optical labels each with increasing wavelengths of emission and excitation, said optical-labels being ordered into pairs with the emission of the first optical-label overlapping the excitation of the second optical-label, the emission of the second optical-label overlapping the excitation of the third optical-label and so forth; the ordering of these monomer units and intermediate monomer units producing a geometry of said optical-labels to maximize energy transfer between the optical-label with the lowest excitation wavelength and the optical-label with the longest emission wavelength.

72. The polymer of Claim 71, wherein said of optical-labels are species emitting light in the range from 300 to 1,400 nanometers after receiving energy in the range from 200 to 1,000 nanometers.

73. The polymer of Claim 49, wherein the sequence of at least two monomer units linked to optical-labels and spacer monomers results in said optical-labels having a relative geometry that maximizes their emissions.

74. The polymer of Claim 48 wherein said tag is an other-label,

75. The polymer of Claim 74, wherein said other-label is radioactive.

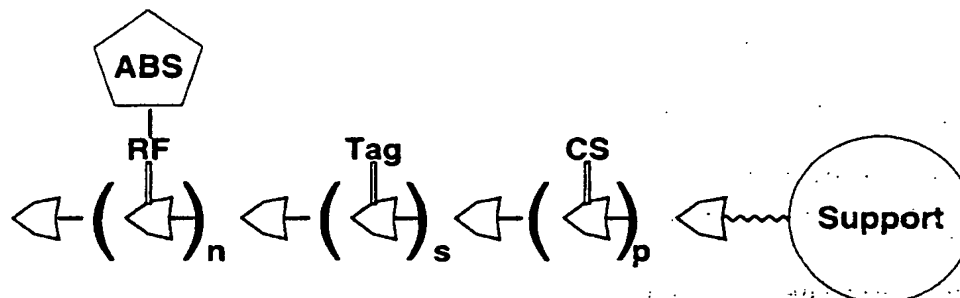
76. The polymer of Claim 74, wherein said other-label is paramagnetic.

77. The polymer of Claim 48 wherein said tag is a separation-tag.

78. The polymer of Claim 77, wherein said separation-tag is a moiety increasing magnetic susceptibility, ionic charge, mass, or density.

79. The polymer of Claim 6, comprising at least two monomer units each linked to a tag, said tags being the same or different and said tagged monomer units being sequentially ordered to control their interactions; and at least one monomer unit linked to an analyte-binding species or an analyte.

80. The polymer of Claim 79 represented by the schematic Formula IX

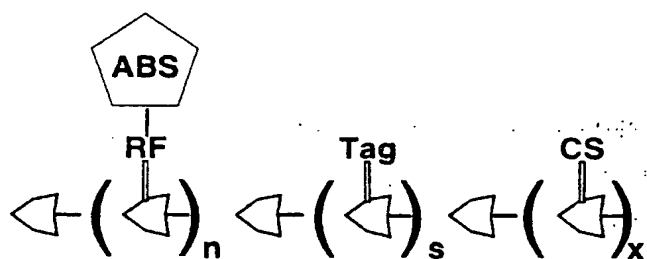


Formula IX

wherein each left pointing broad-arrow shape represents a monomer unit; RF represents a reactive functionality linked to a monomer unit; Tag independently at each occurrence represents an optical-label, other-label, or separation-tag linked to a monomer unit; CS represents a cleavable link to the solid support shown by the circular shape at the right; the pentagon labeled ABS represents an analyte-binding species linked to the polymer by a covalent bond to a monomer unit through a reactive functionality; broad-arrow shapes without other indication represent spacer monomer units,  $n$  is a number from 1 to 10,  $s$  is a number from 2 to 1000, and  $p$  is a number from 1 to 25.

81. The polymer of Claim 79 selectively cleaved from its support.

82. The polymer of Claim 81 represented by the schematic Formula X:



Formula X

wherein, each left pointing broad-arrow shape represents a monomer unit; RF represents a reactive functionality linked to a monomer unit; Tag independently at each occurrence represents an optical-label, other-label, or separation-tag linked to a monomer unit; the pentagon labeled ABS represents an analyte-binding species linked to the polymer by a covalent bond to a monomer unit through a reactive functionality; broad-arrow shapes without other indication represent spacer monomer units, n is a number from 1 to 10, s is a number from 2 to 1000, and x is a number from 1 to 25 and is less than or equal to p of Formula VIII.

83. The polymer of Claim 82 wherein said water-soluble polymer consists primarily of amino acid monomers.

84. The polymer of Claim 83 wherein said amino acid monomers comprise naturally occurring amino acids and synthetic amino acids.

85. The polymer of Claim 83 wherein said amino acid monomers comprise L amino acids and D amino acids.

86. The polymer of Claim 83, comprising at least one amino acid monomer unit absorbing light in the range from 200 to 300 nm.

87. The polymer of Claim 83, comprising at least one monomer unit of tryptophan.

88. The polymer of Claim 82 wherein said water-soluble polymer consists primarily of nucleotide monophosphate monomers.

89. The polymer of Claim 82 wherein said water-soluble polymer consists primarily of sugar monomers.

90. The polymer of Claim 82 wherein said water-soluble polymer consists primarily of any monomer that can have a functionalized side chain and undergo sequential synthesis on a solid support.

91. The polymer of Claim 82 where at least one of said tags is an optical-label.

92. The polymer of Claim 82 where more than one of said tags are optical-labels.

93. The polymer of Claim 92, wherein said optical-labels are capable of absorbing and/or emitting light in the wavelength range from 200 to 1400 nanometers.



94. The polymer of Claim 92, comprising at least two different optical-labeled monomer units.
95. The polymer of Claim 94, wherein a first optical-label is capable of transferring energy to a second optical-label.
96. The polymer of Claim 95, wherein the order of the monomer units is such that said first optical-label has a geometry relative to said second optical label to maximize energy transfer between said optical-labels.
97. The polymer of Claim 95, wherein the order of the monomer units is such that there are three or more optical labels each with increasing wavelengths of emission and excitation; said optical-labels being ordered into pairs with the emission of the first optical-label overlapping the excitation of the second optical-label; and the emission of the second optical-label overlapping the excitation of the third optical-label and so forth; the ordering of these monomer units and intermediate monomer units producing a geometry of these optical-labels to maximize energy transfer between the optical-label with the lowest excitation wavelength and the optical-label with the longest emission wavelength.
98. The polymer of Claim 95, wherein each of said optical-labels is a species having an emission spectrum maximum in the range from 300 to 1,400 nanometers.
99. The polymer of Claim 95, wherein each of said optical-labels is a species having an excitation spectrum maximum in the range from 200 to 1,000 nanometers.
100. The optical-labels of Claim 95 where said first optical-label and second optical-label are an organic optical-label pair capable of energy transfer.
101. The polymer of Claim 91, wherein the order of tagged monomer units having identical optical-labels results in said optical-labels having a relative geometry that maximizes their emissions.
102. The polymer of Claim 91 wherein said optical-label is a lanthanide compound.
103. The polymer of Claim 91 wherein said optical-label is a lanthanide complex.
104. The polymer of Claim 91 wherein said optical-label is a lanthanide macrocycle.
105. The polymer of Claim 102 wherein said lanthanide compound is a compound of europium, samarium, terbium, or dysprosium.

106. A process for the production of a conjugate of an analyte-binding species and a tagged water-soluble, polymer which comprises the following steps:

1. iterative synthesis of a water-soluble polymer linked to a solid support, said polymer including
  - a. at least one functionalized monomer unit that can each selectively covalently bind with a specific functionalized tag, or already has a specific functionalized tag attached;
  - b. at least one reactive functionality able to bond to an analyte-binding species;
  - c. a cleavage segment of at least one monomer unit selectively cleavable from said support;
2. when necessary, specifically reacting functionalized monomer units of said polymer with one or more tags;
3. specifically reacting one or more functionalized monomer units of said polymer with an analyte-binding species; and
4. selectively cleaving the selectively cleavable linkage with the liberation of a tagged water-soluble, polymer conjugate of an analyte-binding species.

107. A process of preparing a tagged water soluble polymer comprising at least two monomer units linked to a reactive functionality and at least one spacer monomer unit, at least one of said monomer units being linked to an optical-label capable of absorbing and/or emitting light, comprising the steps of

1. providing a first monomer having 2-3 reactive functionalities of which one is free and the remainder are protected,
2. reacting the free reactive functionality of said monomer with a suitable support so as to link said monomer to said support which are attached to said support.
3. deprotecting one remaining protected reactive functionality of said monomer,
4. providing a second monomer having 2-3 reactive functionalities of which one is free and the remainder are protected, said first monomer and said second monomer being the same or different,
5. reacting said second monomer with the product of step 3, thereby linking said second monomer to said support through said first monomer,
6. deprotecting one remaining protected reactive functionality of said second monomer,
7. repeating steps 3), 4) and 5) with additional monomers having 2-3 reactive functionalities of which one is free and the remainder are protected, said additional monomers being the same as said first and/or second monomer, or different, thereby linking said additional monomers in predetermined number and sequence to said support through said first monomer and said sec-

ond monomer to yield a polymer comprising units of monomers in the number and sequence in which they have been reacted and linked to said support,

8. selectively cleaving said polymer from said support, and

9. deprotecting remaining protected reactive functionalities, wherein at least one monomer is linked to an optical-label capable of absorbing and/or emitting light at a wave-length of 200 to 1400 nanometers.

108. The process of Claim 107, wherein at least one monomer is an alpha-aminocarboxylic acid linked to a reactive functionality.

109. The process of Claim 107, wherein at least one monomer is a spacer alpha-aminocarboxylic acid.

110. The process of Claim 107, wherein the polymer has a molecular weight in the range of 1,000 to 100,000 daltons.

111. The process of Claim 107, wherein said optical-label is a heterocyclic macrocycle having a lanthanide metal central atom.

112. The process of Claim 111, wherein said central atom is selected from the group consisting of dysprosium, europium, samarium, and terbium.

113. The process of Claim 107, wherein said selective cleavage is carried out in presence of an enzyme.

114. The process of Claim 107, carried out a temperature between -10° C and 50° C.

115. The process of Claim 107, carried out a a pH ranging from 6 to 9.

116. A method for detecting an analyte comprising the steps of linking the analyte to an analyte-binding species linked to a polymer according to Claim 6 having a tag that is an optical-label, and measuring the light absorption and/or emission of said optical-label before or after cleavage of said polymer from said solid support.

117. The method of Claim 116 wherein the analyte is a hapten having a molecular weight in the range of 125-2000 daltons.

118. The method of Claim 116 wherein the analyte has a molecular weight greater than 2000 daltons.

119. The method of Claim 117 in which the hapten is selected from the group consisting of

- (a) Vitamins, vitamin precursors, and vitamin metabolites including retinol, vitamin K, cobalamin, biotin, folate;
- (b) Hormones and related compounds including
  - (i) steroid hormones including estrogen, corticosterone, testosterone, ecdysone,
  - (ii) aminoacid derived hormones including thyroxine, epinephrine,
  - (iii) prostaglandins,
  - (iv) peptide hormones including oxytocin, somatostatin,
- (c) pharmaceuticals including aspirin, penicillin, hydrochlorothiazide,
- (d) Nucleic acid constituents including
  - (i) natural and synthetic nucleic acid bases including cytosine, thymine, adenine, guanine, uracil, derivatives of said bases including 5-bromouracil,
  - (ii) natural and synthetic nucleosides and deoxynucleosides including 2-deoxyadenosine, 2-deoxycytidine, 2-deoxythymidine, 2-deoxyguanosine, 5-bromo-2-deoxyuridine, adenosine, cytidine, uridine, guanosine, 5-bromouridine,
  - (iii) natural and synthetic nucleotides including the mono, di, and triphosphates of 2-deoxyadenosine, 2-deoxycytidine, 2-deoxythymidine, 2-deoxyguanosine, 5-bromo-2-deoxyuridine, adenosine, cytidine, uridine, guanosine, 5-bromouridine,
- (e) drugs of abuse including cocaine, tetrahydrocannabinol,
- (f) histological stains including fluorescein, DAPI
- (g) pesticides including digitoxin,
- (h) and miscellaneous haptens including diphenylhydantoin, quinidine, RDX.

120. The method of Claim 118 in which the analyte is selected from the group consisting of

- (a) polyaminoacids, polypeptides, proteins, polysaccharides, nucleic acids, glycosaminoglycans, glycoproteins, ribosomes and
- (b) proteins and their combinations including
  - (i) albumins, globulins, hemoglobin, staphylococcal protein A, alpha-fetoprotein, retinol-binding protein, avidin, streptavidin, C-reactive protein, collagen, keratin,
  - (ii) immunoglobulins including IgG, IgM, IgA, IgE,
  - (iii) Hormones including lymphokines, follicle stimulating hormone, and thyroid stimulating hormone,
  - (iv) enzymes including trypsin, pepsin, reverse transcriptases
  - (v) cell surface antigens on T- and B-lymphocytes; i.e. CD-4, CD-8, CD-20 proteins, and the leukocyte cell surface antigens, such as described in the presently employed CD nomenclature;
  - (vi) blood group antigens including A, B and Rh,
  - (vii) major histocompatibility antigens both of class 1 and class 2,
  - (viii) hormone receptors including estrogen receptor, progesterone receptor, and glucocorticoid receptor,
  - (ix) cell cycle associated proteins including protein kinases, cyclins, PCNA, p53,
  - (x) antigens associated with cancer diagnosis and therapy including BRCA(s) carcinoma embryonic antigen, HPV 16, HPV 18, MDR, c-neu; tumor suppressor proteins, p53 and retinalblastoma,
  - (xi) apoptosis related markers including annexin V, bak, bcl-2; fas caspases, nuclear matrix protein, cytochrome c, nucleosome,
  - (xii) toxins including cholera toxin, diphtheria toxin, and botulinum toxin, snake venom toxins, tetrodotoxin, saxitoxin,
  - (xiii) lectins including concanavalin, wheat germ agglutinin, soy bean agglutinin,

- (c) polysialic acids including chitin;
- (d) polynucleotides including
  - (i) RNAs including segments of the HIV genome, human hemoglobin A messenger RNA,
  - (ii) DNAs including chromosome specific sequences, centromeres, telomere specific sequences, single copy sequences from normal tissues, single copy sequences from tumors.

121. The method of Claim 116 in which said optical-label is an organic optical-label.

122. The method of Claim 121 in which said optical-label is selected from the group consisting of fluorescent-labels.

123. The method of Claim 121 in which said fluorescence-label is selected from the group consisting of: acridine, bodipy, cyanine, oxazine, oxazole, pyrene, styryl, thiazole, fluorophores; fluorescein and its derivatives; rhodamine and its derivatives, coumarin and its derivatives; naphthalene and its derivatives, porphyrins.

124. The method of Claim 121 in which said optical-label is selected from the group consisting of absorbance-labels.

125. The method of Claim 121 in which said absorbance-label is selected from the group consisting of: alizarin, alcian, amido, aniline, astrazone, auramine, azine, azo, azul, benzamine, benzo, benzyl, biebrich, bodipy, brentamine, chlorantine, chlorazole, chrysoidine, coomassie, cyanine, dianil, diazo, durazol, eosin, eriochrome, fuchsin, janus, lissamine, naphthalene, naphthol, oxazine, oxazole, ponceau, pyrene, pyronine, rosaniline, sirus, solochrome, stilbene, styryl, sudan, supramine, supranol, thiazole, thionine, toluidine, triarylmethane, triphenylmethane, trisazo, trisulphon, trypan, xylene, xylidine, and zapon dyes; fluorescein and its derivatives; rhodamine and its derivatives; coumarin and its derivatives; naphthalene and its derivatives.

126. The method of Claim 116 in which said optical-label is a lanthanide compound.

127. The method of Claim 116 in which said optical-label is a lanthanide complex.

128. The method of Claim 116 in which said optical-label is a lanthanide macrocycle.

129. The method of Claim 126 in which said lanthanide compound is a compound of europium, samarium, terbium, or dysprosium.

130. A process of preparing a polymer according to Claim 82 comprising at least two monomer units linked to an analyte binding species and at least one spacer monomer unit, at least two of said monomer units being amino acids linked to an optical-label capable of absorbing and/or emitting light, comprising the steps of

1. modeling a sequence of amino acids to optimize the distance and geometry between fluorescence-labeled monomers;
2. synthesizing with the technology of combinatorial chemistry multiple polymers;
3. screening said multiple polymers by their fluorescence and/or luminescence spectra for maximum desired emission to determine potential candidates to be optical-labels in tagged-peptides;
4. synthesizing polymers linked to a solid support of Claim 1;
5. coupling an analyte binding species to said polymers;
6. cleaving the optically labeled polymers of Claim 54;
7. and utilizing the optically labeled polymer analyte binding species conjugates according to the methods of Claim 116.

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/27787

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(7) : G01N 33/545, 33/543, 33/576, 33/539; C08F 2/10, 2/50, 290/14;

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7, 7.21, 7.24, 29, 34, 39; 436/17, 63, 519, 536, 546; 250/461.2; 356/39, 424/3, 7.1;

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
STN- biosis, caplus, embase, medline, aisline, cancerlit; EAST and WEST-patent database**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 95/02184 A1 (BAXTER DIAGNOSTICS INC.) 19 January 1995, see entire document.	1-130
Y	US 4,647,598 A (YADA et al.) 03 March 1987, see entire document.	1-130

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"A" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

14 DECEMBER 2000

Date of mailing of the international search report

29 JAN 2001

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

LISA V. COOK

Telephone No. (703) 308-0196



# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US00/27787

## A. CLASSIFICATION OF SUBJECT MATTER: US CL :

435/7, 7.21, 7.24, 29, 34, 39; 436/17, 63, 519, 536, 546; 250/461.2; 356/39, 424/3, 7.1;